T-cell response against human malignant melanoma.

Thesis

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PER THOR STRATEN

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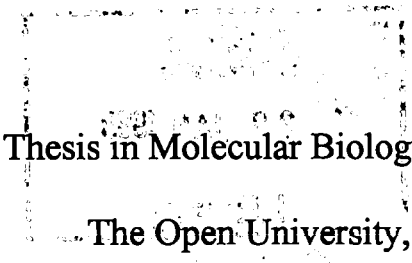

Ph.D. Thesis in Molecular Biology by Per thor Straten, MSc

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Abstract

The present Thesis is introduced with a discussion of reverse transcription polymerase chain reaction (RT-PCR) based analysis of TCRBV regions, including the description of a panel of primers. Using this primer panel, we analysed for TCR clonality in progressive *versus* regressive parts of partially regressive primary melanoma lesions (Paper 1). The main conclusion drawn from this study is that clonotypic T cells were present in both regressive and progressive parts of the same lesions. However, sequencing of "non clonal" BV-regions demonstrated that most of them were not polyclonal by nature but oligoclonal e.g. that in some cases all transcripts were of the same length or only a limited number of BJ regions were used. This indicated that some T-cell clones were left undetected by direct sequencing.

To facilitate full and detailed analysis of T-cell clonotypes in the infiltrates we aimed at establishing a suitable method for the detection of clonally expanded T cells. Denaturing gradient gel electrophoresis (DGGE) was chosen because this method is highly sensitive and excludes laborious steps that are obligatory in almost all other methods dealing with the detection of TCR clonality (Paper 2).

We applied the DGGE based method to analyse for *in situ* T-cell clonality in 6 subcutaneous melanoma lesions from two patients. The results demonstrated two important characteristics of *in situ* TIL in melanoma. First, the infiltrate constitutes an exceedingly high number of different T-cell clones, and second, the T-cell response appears to be executed by local T cells that do not enter the periphery (Paper 3).

The final study deals with a comparative analysis of *in situ* T cells and T cells propagated *in vitro*, demonstrating that standard *in vitro* culture conditions do not support the growth of *in vivo* expanded T-cell clones (Paper 4).

List of papers or manuscripts included in the Thesis.

1. thor Straten, P., Becker, J.C., Seremet, T., Bröcker, E.B., and Zeuthen, J. Clonal T-cell responses in tumour infiltrating lymphocytes from regressive and progressive regions of primary human melanoma. J. Clin. Invest., *98*: 279-284, 1996.

2. thor Straten, P., Barfoed, A., Seremet, T., Säterdal, I., Zeuthen, Z., and Guldberg, P. Detection and characterisation of αβ T-cell clonality by denaturing gradient gel electrophoresis (DGGE). Biotechniques, *In press*.

3. thor Straten, P., Guldberg, P., Riise Hansen, M., Kirkin, A.F., Seremet, T., E. Siim, Zeuthen, J., and Becker, J. C., Local versus systemic immune reactions against disseminated melanoma: predominance of localised clonotypic T cell expansions. *Manuscript in preparation.*

4. thor Straten, P., Guldberg, P., Riise Hansen, M., Kirkin, A.F., Seremet, T., Dahlström, K., Drzewiecki, K., Becker, J. C., and Zeuthen, J. Clonotypic T cell expansion induced by melanoma: Comparison of the *in vivo* and *in vitro* situations. *Submitted.*

List of papers not included in the Thesis

1. Andersen, E., Schøller, J., thor Straten, P., Dunn, S., and Zeuthen, J. T-cell receptor Vα and Vβ gene usage in interleukin-2-cultured tumor infiltrating lymphocytes from patients with breast cancer. Int. J. Oncol., *4*: 1035-1041, 1994.

2. thor Straten, P., Schøller, J., Hou-Jensen, K., and Zeuthen, J. Preferential usage of T-cell receptor αβ-variable regions among tumor-infiltrating lymphocytes in primary human malignant melanomas. Int. J. Cancer, *56*: 78-86, 1994.

3. Schøller, J., thor Straten, P., Birck, A., Siim, E., Dahlstrøm, K., Drzewiecki, K.T., and Zeuthen, J. Analysis of T cell receptor αβ variability in lymphocytes infiltrating melanoma primary tumours and metastatic lesions. Cancer Immunol. Immunother., *39*: 239-248, 1994.

4. Kirkin, A.F., Reichert Petersen, T., Olsen, A.C., Li, L., thor Straten, P., and Zeuthen, J. Generation of human-melanoma specific T lymphocyte clones defining novel cytolytic targets with panels of newly established melanoma cell lines. Cancer Immunol. Immunother., *41*: 71-81, 1995.

5. Zeuthen, J., Birck, A., and thor Straten, P. Analysis of T cell receptor AB variability in tumor infiltrating lymphocytes in primary and metastatic melanoma. Arc. Imm. Ther. Exp., *43*: 1-15, 1995.

6. Kirkin, A.F., thor Straten, P., and Zeuthen, J. Differential modulation by interferon gamma of the sensitivity of human melanoma cells to cytolytic T-cell clones that recognise differentiation or progression antigens. Cancer Immunol. Immunother., *42*: 203-212, 1996.

7. Birck, A., Li, L., thor Straten, P., Zugar, J., Hou-Jensen, K., and Zeuthen, J. Analysis of T-cell receptor AV and BV chain variable gene expression by infiltrating lymphocytes in Spitz and Halo Nevi. Melanoma Res., *7*: 49-57, 1997.

8. Guldberg, P., Kirkin, A.F., Grønbæk, K., thor Straten, P., Ahrenkiel, V., and Zeuthen, J. Complete scanning of the CDK4 gene by denaturing gradient gel electrophoresis: A novel

missense mutation but low overall frequency of mutations in malignant melanoma. Int. J. Cancer, *72*: 780-783, 1997.

9. Guldberg, P., thor Straten, P., Birck, A., Ahrenkiel, V., Kirkin, A.F., and Zeuthen, J. Disruption of the MMAC1 gene by deletion or mutation is a frequent event in malignant melanoma. Cancer Res., *57*: 3660-3663, 1997.

10. thor Straten, P., Kirkin, A.F., Seremet, T., and Zeuthen, J. Expression of transporter associated with antigen processing 1 and 2 (TAP1/2) in malignant melanoma cell lines. Int. J. Cancer, *70* : 582-586, 1997.

11. Guldberg, P., Grønbæk, K., Aggerholm, A., thor Straten, P., Ahrenkiel, V., Hokland, P., and Zeuthen, J. Detection of mutations in GC-rich DNA by bisulphite denaturing gradient gelelectrophoresis. Nucleic Acids Res., *26*: 1548-1549, 1998.

12. Säterdal, I., thor Straten, P., Mykleburst, J.H., Kirkin, A.F., Gjertsen, M.K., and Gaudernack, G. Generation and characterisation of GP100 peptide specific NK-T cell clones. Int. J. Cancer, *75*: 794-803, 1998.

13. thor Straten, P., Ralfkiaer, E., Hendriks, J., Seremet, T., Vejlsgaard, G.L., and Zeuthen, J. T-cell receptor variable region genes in cutaneous T-cell lymphomas. Br. J. Dermatol., *138*: 3-12, 1998.

14. thor Straten, P., Guldberg, P., Seremet, T., Reisfeld, R., Zeuthen, J., Becker, J. C. Activation of pre-existing T-cell clones by targeted interleukin 2 therapy. Proc. Natl. Acad. Sci. USA *95*: 8785-8790, 1998.

15. Becker, J.C., Guldberg, P., Bastian, B., Terheyden, P., Seremet, T., Siedel, C., Zeuthen, J., Bröcker, E. B., Zeuthen, J. and thor Straten, P. *In vivo* evidence for anti-self- and anti-tumor-immunity exerted by the same T-cell clone. *Submitted.*

16. Dzhandzhugazyan, K., Kirkin, A. F., thor Straten, P., Zeuthen, J. Ecto-ATP diphosphohydrolase/CD39 is overexpressed in differentiated human melanomas. FEBS Letters, *430*, (3): 227-230, 1998.

17. Grønbæk, K., thor Straten, P., Ralfkiaer, E., Ahrenkiel, V., Klarskov Andersen, M., Hansen, E. B., Zeuthen, J., Hou-Jensen, K., Guldberg, P. Somatic *Fas* mutation in Non-

Hodgkin's lymphoma: Association with extra-nodal disease and autoimmunity. Blood *92*, 3018-3024, 1998.

18. Guldberg, P., thor Straten, P., Ahrenkiel,V., Seremet, T., Kirkin, A. F., Zeuthen, J. Somatic mutation of the Peutz-Jeghers syndrome gene, STK11/LKB1, in malignant melanoma. Oncogene, *in press*.

19. Grønbæk K., Nedergaard, T., Andersen, M. K., thor Straten, P. Guldberg, P. Møller, P., Zeuthen, J., Ebbe Hansen, N., Hou-Jensen, K., Ralfkiaer, E. Concurent disruption of cell cycle associated genes in mantle cell lymphoma: a genotypic and phenotypic study of cyclin D1, p16, p15, p53 and pRb. Leukemia, 12, 1266-1271, 1998.

Preface and acknowledgements.

The work summarised in the present Thesis was carried out in the years 1995-1998 at the Department of Tumor cell Biology, The Danish Cancer Society, Strandboulevarden 49, 2100 Copenhagen, Denmark. The work was supervised by Professor Jesper Zeuthen, Head of Department, Department of Tumor Cell Biology, The Danish Cancer Society and Professor P. Moss, C.R.C. Institute for Cancer Studies, University of Birmingham, Birmingham B13 2TA, UK.

The present Thesis is compiled of four studies. Although there is some methodological overlap from one study the other, each study can be read more or less independently.

Four persons have been deeply involved in the present work; Jesper Zeuthen, Jürgen C. Becker, Per Guldberg and Tina Seremet.

I first met Jesper in 1991 when I was looking for a laboratory to do my Masters Degree. Jesper was at that time just about to take on a project on tumour infiltrating lymphocytes (TIL) in melanoma and together with senior scientist Jørgen Schøller I participated in that project from the very beginning. I finished my Masters Degree in 1993 and soon after Jørgen Schøller left the group. Being the only biologist in the laboratory, Jesper let me continue working on the project. Through all that time that went by before things (publications) slowly began to happen, I never heard a sour word or a bitter comment from Jesper. On the contrary, even the smallest of successes were lifted to the sky. I don't think that anyone except Jesper could have stayed optimistic and patient through all that time. But I am glad and grateful that *he* did.

I met Jürgen C. Becker in Lyon in 1993 at an EORTC melanoma group meeting. Jürgen was at that time doing clinical work at the Department of Dermatology at the University Hospital in Würzburg, and doing part-time research in the basement after working hours. And doing well. The data from our first collaboration were published in *Journal of Clinical Investigations*, and I was thrilled. A paper like that was above my wildest

imagination. Knowing Jürgen a whole lot better know, my imagination no longer has such limits. Maybe part of it is related to the Havana Club, but things always turn out great when Jürgen is involved.

I first met Per Guldberg at the University following a course in genetics. We did a project on yeast genetics together and Per kept correcting my writing. I was sick and tired of it. However, after the examination I had to admit that he was right and I was wrong. He still corrects my writing and he is still right. (Even) more scientifically Per introduced mutation detection by denaturing gradient gel electrophoresis (DGGE) in the laboratory. He tried to convince me to try the DGGE method for detection of T-cell clones for so long that it's a mystery he didn't give up. When I finally gave in we started out analysing TILs of B16 tumours; a study we did together with Jürgen. The first gel was loaded with bands; each band corresponding to a clonotypic TCR transcript – I almost didn't believe it. These first data were published in *Proceeding of the National Academy of Sciences, USA*. Without hesitation the method was established for the human TCRs – as it will appear, the present Thesis would be very different without the DGGE method. Needless to say, the DGGE methodology moved things a lot, and I expect a lot of it.

Tina Seremet has been working together with me for the past five years. Doing a great job. In fact doing so great that it has been hard to find the time to write this Thesis. New data is piling up on my desk and I don't know how I will ever work my way through it.

Thanks for proof reading, good ideas, hints, figures, and discussions to Annette Barfoed, Jes Forchammer, John Haurum, Mai-Britt Nielsen, Jes Dietrich, Aleixei Kirkin, Mikkel Rohde, and first and foremost Jesper, Jürgen and Per.

Also thanks to everyone in the laboratory; Kisser, Vibs, Dr. Worm, Lillan, Cat-woman, Anette Birch, Mia, Dorrit Lützhøft, Said, Fisher-pigen, Marianne, Karine, Jaqueline, Eduardo, Natalia, Dorte, Mads, Ingelise, Zsofia, Tina, Merete, and, and, and.... Hopefully I didn't forget anyone.

Authorship declaration

The work described in the present Thesis is my own with the following exceptions.

Annette Barfoed and Alexei F. Kirkin, both at the Department of Tumour Cell Biology, The Danish Cancer Society, performed cell culture.

Jürgen C. Becker, Department of Dermatology, University Hospital, Würzburg, Germany, performed Immunohistology.

Surgery of melanoma patients was performed by Drs. Karin Dahlström, Krzysztof T. Drzewiecki, and Elsebeth Siim at the Department of Plastic and Reconstructive Surgery, State University Hospital, Copenhagen, Denmark (paper 3, patient #1) and Jürgen C. Becker, University Hospital, Würzburg, Germany (paper 1, all patients and paper 3, patient #2).

Dorrit Lützhøft performed preparation of frozen samples for RNA isolation.

Contents

Introduction to the cell mediated immune system

The vertebrate body offers an ideal breeding ground for a variety of virus and bacteria. The immune system deals with this challenge in a controlled fashion, clearing the body for virulent pathogens. The immune system can roughly be divided into innate and adaptive branches. Whereas the innate immune system in general recognises posttranslational modifications on infected cells or on pathogens themselves, adaptive immune responses are characterised by the specific receptor mediated recognition of antigen or antigen fragments. In general, adaptive immunity is considered as a second line of defence, dealing with the pathogens that are not cleared from the body by innate immunity.

Another characteristic of adaptive immunity is the capacity to memorise and respond rapidly to a second exposure to antigen. B cells and T cells are the most important cell types of adaptive immunity. B cells control humoral immunity by the production of antibodies (Ab) whereas T cells are involved in a much broader spectrum of cellular immunity. Where B cells recognise native antigen, T cells are restricted to the recognition of antigen fragments presented in the context of MHC molecules. As T-cell responses are the main topic of this Thesis, the following introduction will focus on this cell type.

The Major Histocompatibility Complex (MHC)

The initiation of a T-cell response is dependent upon the processing and presentation of the antigenic peptide in the context of Major Histocompatibility Complex (MHC) class I or class II molecules. Most nucleated cells express class I molecules, whereas class II molecules are expressed mainly by specialised antigen presenting cells (APC): macrophages, B cells, and dendritic cells (DCs). In general peptides derived from intracellular antigens are presented to CD8 positive ($CD8^+$) T cells in the context of class I molecules, while peptides derived from extracellular antigens are presented to CD4 positive ($CD4^+$) T cells in the context of class II molecules.

The MHC genes are polygenic. This means that there are several different MHC class I and class II genes, encoding proteins with different peptide specificities. Furthermore, the MHC genes are the most polymorphic genes known, implying that each MHC gene exists as several allelic variants.

Class I MHC: Structure and peptide presentation

Cells of higher vertebrates have evolved a highly complex enzymatic system that allows intracellular proteins to be processed into small peptide fragments and presented on the cell surface, available for immune surveillance. The presentation of these peptides occurs in the context of MHC class I molecules which consist of two non-covalently associated proteins, the membrane spanning glycosylated α-chain (heavy chain; HC) and the smaller β_2-microglobulin (β_2M). The Class I molecules in humans are encoded by three class I α-chain genes (HLA-A, -B, and –C) and have four domains; the β_2M and three domains of HC, α_1, α_2, and α_3 of which α_1 and α_2 together form the peptide binding groove (1). Both the β_2M and the HC are co-translationally inserted into the endoplasmatic reticulum (ER) in a signal-sequence dependent fashion upon which the HC assembles with the membrane bound chaperone calnexin until complexing with β_2M (2). Another chaperone, calreticulin, binds the class I molecule prior to peptide loading, the peptides being translocated to the lumen of ER from the cytosol by the transporters associated with antigen processing (TAP). During the association of the class I molecule and TAP which is facilitated by the ER resident protein tapasin,

peptides are loaded onto the class I molecule (3). Peptide binding stabilises the MHC molecule and induces its release from the ER after which the class I molecule migrates to the surface (4).

The peptides binding to class I molecules encompass 8-12 amino acids. Cytosolic proteins are targeted for degradation by the attachment of ubiquitin making them susceptible for degradation by the proteasome complex (2).

Class II MHC: Structure and peptide presentation

MHC class II molecules in general bind and present peptides derived from exogeneous proteins. The class II molecule is composed of two transmembrane glycoprotein chains, the α- and the β-chain. As for the class I genes there are three pairs of α- and β chain genes, HLA-DR, -DP, and DQ. However, the HLA-DR locus encodes an additional β-chain protein which can pair with the DRα chain implying that the three sets of class II genes may give rise to four different class II molecules (5). Furthermore, the possible paring of paternal and maternal class II chains adds to the number of different Class II molecules.

Each chain of the class II molecule has two domains, α_1 and α_2 (for the α-chain) and β_1 and β_2 (for the β chain), together forming a four-domain structure similar to the class I molecule. However, the peptide binding groove of the class II molecule is open at both ends, and the peptides binding to the class II molecule are generally at least 13 amino acids in length but may be much longer. Prior to binding of peptide, the class II molecule assembles in the ER with the Invariant chain (Ii) making a nonameric complex $(\alpha\beta Ii)_3$ in which the peptide binding groove of the class II molecule is occupied by the CLIP region of the Ii. The presence of the invariant chain directs the complex to the endosomes, in which exogenous proteins are degraded after capture from the environment by receptor mediated endocytosis, phagocytosis or macropinocytosis (6). The removal of the CLIP peptide and the favoured binding of peptides with high affinity are facilitated by the MHC encoded HLA-DM molecule (7; 8). After assembly in the endosome the class II MHC/peptide complexes traffic to the cell surface, although the route by which the class II molecule reaches the cell surface is not fully understood (6).

The T cell

T-cell functions

The function of T cells is to recognise the presence of pathogens in the body and to respond accordingly, either by direct elimination of the pathogen or by activating other parts of the immune system to clear the pathogen. T cells do not recognise native antigen but small antigen fragments (peptides) presented in the context of MHC molecules. This dual specificity for both MHC and antigen is accomplished by the T-cell receptor (TCR), consisting of an αβ- or a γδ-heterodimer (9).

T cells constitute approximately 70 % of the peripheral blood lymphocytes in adult humans and are responsible for the majority of the cell mediated immune responses that depend on cell to cell interactions. The main types of T cells are the cytotoxic T cells (CTL), and T helper cells (Th). In general these T-cell subsets can be distinguished phenotypically by the expression of the CD4 or the CD8 molecules, the CD8 molecule being expressed by the CTL, whereas the CD4 molecule is expressed on Th cells.

The most direct function of the different T-cell types is exerted by the CTL which recognise endogeneous antigens, in general derived from intracellular pathogens. Upon recognition and activation CTL are capable of killing the infected cell. Killing of the target cell may be accomplished by the granule exocytosis mechanism, in which release of cytotoxins will generate pores in the target cell membrane. Cytotoxicity may also be induced by ligation of Fas ligand (FasL,CD95L) on the effector cell with trimeric Fas (CD95) on the target cell generating a death signal that induces apoptosis (10).

The Th cells can be divided into (at least) two different groups; the Th1 cells (or inflammatory T cells) and the Th2 cells. Th1 and Th2 cells in general recognise peptides derived from exogeneous proteins presented in the context of class II MHC molecules. The actions of Th1 and Th2 cells are exerted by means of cytokine production by which the Th1 cells activate macrophages during inflammation (by secretion of interleukin (IL) 2, interferon-β (IFN-β), and interferon-γ (IFN-γ)), whereas Th2 cells activate B cells by the secretion of IL-4, IL-5, IL-6 and IL-10 (11).

T-cell development

T cells are derived from pluripotent stem cells in the bone marrow. Committed T-cell precursors migrate to the thymus where maturation and rearrangement of the TCR gene segments take place. Early T-cell development is characterised by expansion and differentiation of thymocytes which do not yet express mature TCRs on their cell surface. Immature pro-thymocytes arriving at the thymus express neither the CD4 nor the CD8 molecule ($CD4^-CD8^-$; double negative (DN)). During later steps of T-cell development both CD4 and CD8 are expressed ($CD4^+CD8^+$; double positive (DP)) and the T cell gets committed to express the γδ or the αβ TCR, and to lose the expression of either the CD4 or the CD8 molecule (Fig. 1). Most γδ T cells are, however, of DN phenotype and the mechanisms underlying the commitment to the αβ or the γδ lineage are not fully delineated although successful γδ rearrangement probably influences the lineage fate (12).

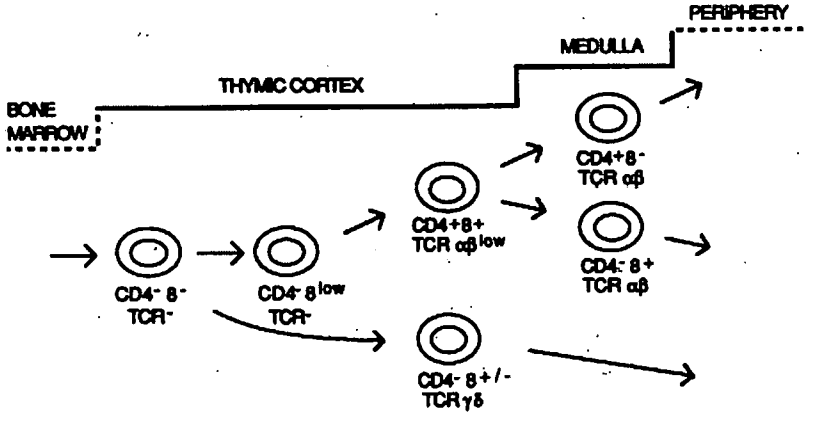

Figure 1. T-cell development and differentiation in the thymus (13).

T cells that react against self-peptides are being eliminated in the thymus, a process referred to as negative selection. In a second selection step in the thymus, TCRs are positively selected for low affinity against MHC/self-peptides. Like with the negative selection, T cells that are unable to exceed a positive affinity/avidity threshold undergo apoptosis (13-15). Only approximately 2-5% of the T cells that enter the thymus go successfully through the steps of rearrangement and selection and subsequently reach the periphery as naïve T cells (13; 16).

The TCR and TCR rearrangement

Antigen recognition by T cells is mediated through the TCR, a clonally distributed heterodimer, in most T cells consisting of an α and a β chain, although a small fraction uses a γδ heterodimer instead (9). Both the α and β chains are glycoproteins with molecular weights between 40 and 60 kD. Each T cell expresses a clonotypic TCR on the cell surface together with the non-clonotypic members of the TCR signaling complex: the different chains of the CD3 complex (γ, δ, ε) and the ζ chain homodimer (Fig. 2) (17).

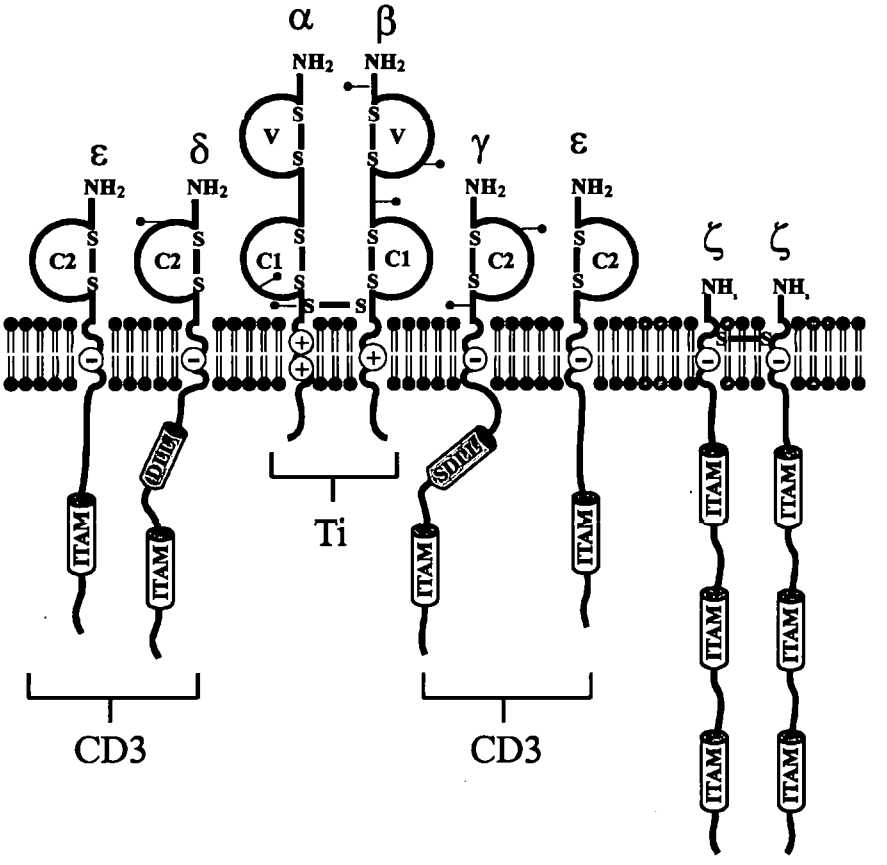

Figure 2. Components of the T-cell receptor: The αβ-TCR together with the γ, δ, and ε chains of the CD3, and the ζ-homodimer (18).

In the germline, each of the TCR proteins is encoded by variable (V), joining (J) and a constant (C) gene segment(s), and for the β chain, an additional diversity (D) segment (13). During T-cell maturation in the thymus the α and β or the γ and δ gene-segments are rearranged into a single transcriptional unit for each of the chains, encoding unique αβ or γδ TCR genes in each T cell. During

the rearrangement process, mediated by at least two proteins (RAG-1 and RAG-2) (19), the V, J, and D segments recombine to form a single exon adjacent to the C segment (Fig. 3).

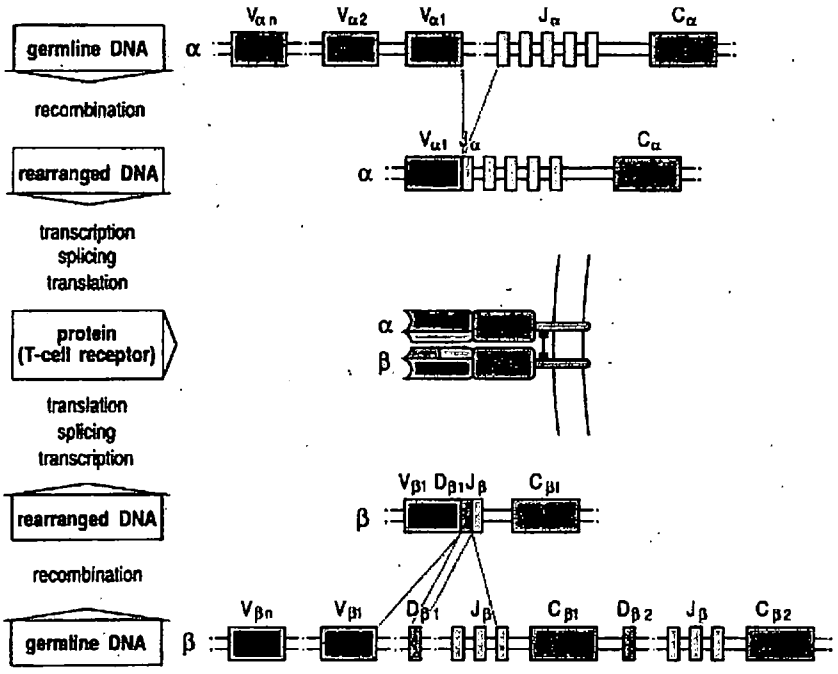

Figure 3. Somatic rearrangement at the TCR α and β locus (5).

In developing T cells TCR rearrangement is initiated at one of the β alleles, and continues on the other only when the first attempt is unsuccessful (20). This event in T-cell development is controlled by a pre-TCR complex consisting of a conventional TCR β-chain and the non-polymorphic pre-TCRα (pTα chain) which is noncovalently associated with components of CD3. The TCRβ/pTα T cells undergo substantial proliferation and develop into the DP phenotype. Subsequently, the TCRα chain is rearranged and the TCRαβ is expressed on the cell surface. The TCRα rearrangements proceed on both chromosomes and are not halted by productive TCRα rearrangement unless the TCRαβ binds to a ligand (21), implying that a T cell may express one TCRβ chain and two TCRα chains (22).

A limitless repertoire for antigen recognition is provided by the assortment of the various V, J, and D gene segments, as well as the addition or deletion of nucleotides at the junction between the

V/J and the J/D gene segments. This so-called "N-region diversity" provides a unique DNA sequence for each individual T cell, forming a clonotype (13). The numbers of gene segments for the α and β chains are given in Table 1. Based on homology (75% at the nucleotide level) the different Vα (AV) and Vβ (BV) segments have been confined into 32 AV families and 26 BV families. Whereas the AV families mostly consist of single member subfamilies, the BV families mainly consist of multi membered families (23), and in general there is a higher degree of homology between the different BV families than between the AV families.

Table 1. The numbers of gene segments encoding the αβ-TCR

	TCR	
	α	β
Variable segments	81	64
Diversity segments	0	2
Joining segments	61	13
Constant segments	1	2

The numbers of the different α and β gene segments are taken from (24; 25).

T cell recognition of antigen

Simple amino acid sequence comparisons years back suggested that TCR heterodimers were Ig-like in structure, including regions with homologies to the Ig complementarity determining regions (CDR) 1, 2, and 3 (26). Early studies proposed that the CDR1 and CDR2 were the parts of the TCR responsible for interaction with the MHC molecule whereas the V-(D)-J region, equivalent to the Ig CDR3, contacts the peptide (27-29). Recently, the crystal structure of the TCR showed that this is not completely correct. The orientation of the TCR to the MHC/peptide complex reveals an interaction in which potentially all CDRs may in fact interact with the peptide, the MHC α-helices, or both, indicating that the CDR3 may not in all cases be the most prominent region for contact to the peptide (30; 31).

In contrast to the high affinity interactions for antibody to antigen, the TCR/MHC-peptide interactions are of low affinity, in the range of 10^{-4} to 10^{-7} M (32). Additionally, part of the affinity in the case of the TCR is directed against the MHC molecule and is not directly related to the antigenic peptide. However, the low affinity of the interaction is measured as soluble complexes. The biological cell to cell affinity is indisputably much higher. The TCR/MHC-peptide interaction is characterised by a very short half life (33), although the cellular interactions between the T cell and the target cell may last for hours during which the T cell polarises the TCRs against the target cell and subsequently downregulates the TCR. Additionally, it has been shown that very few MHC/peptide complexes may induce T-cell activation (33-35), pointing to the model of serial triggering for T-cell activation (34). According to this model, the TCR encounters the MHC/peptide for a short time, reaches an activation threshold upon which the TCR is removed, and another TCR becomes activated by interaction with the MHC/peptide complex (34). Therefore, most likely, the ability of an MHC/peptide complex to induce activation in the T cell is depending on the kinetics of the MHC/TCR interaction in combination with the number of TCRs on the T-cell surface and the number of ligands on the target cell (33; 34). Furthermore, as the kinetics of the TCR/MHC interaction is an important factor, co-receptors CD4 and CD8 may modulate the outcome of the recognition of antigen (36).

T-cell activation

Neither the α nor the β chain of the TCR has large cytoplasmatic domains that might actively participate in intracellular signal transduction. Instead, the ligation of the TCR with the MHC/peptide complex leads to clustering of the TCR/CD3 together with CD45 and CD4 or CD8. Signal transduction is initiated by the activation of protein tyrosine kinases (PTK) of the src and syk families. $p56^{lck}$ tyrosine kinase is associated with CD4/CD8 that become aggregated with the TCR/CD3 upon activation, and $p59^{fyn}$ becomes associated with the CD45 molecule that likewise co-localises with the TCR/CD3 complex. This close encounter enables the $p56^{lck}$/ $p59^{fyn}$ mediated phosphorylation of tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAM) of the CD3/ζ chains (37). ITAMs are present in each of the CD3 subunits; once in the CD3 chains γ, δ and ε, and three

times in each of the two ζ-chains. The phosphorylation of ITAMs facilitate activation of the zeta-associated protein 70 (ZAP-70). The activation of $p56^{lck}$, $p59^{fyn}$ and Zap70 leads to the phosphorylation and activation of several proteins, in turn initiating a number of different signaling pathways (37). The nuclear targets for these signaling pathways are transcription factors, i.e., AP-1, NFAT (38), c-jun and ATF-2 (39; 40).

The naïve T cell requires a co-stimulatory signal in order to reach a state of proliferation and clonal expansion. The requirement of a co-stimulatory signal for T-cell activation confers a mode in which T-cell responses may be controlled. Naïve T cells that recognise antigen expressed on a cell that cannot provide additional co-stimulation become anergic and unable to produce IL-2 (41) even upon subsequent antigen presentation by APC (42). The co-stimulatory signal is provided by ligation of CD28 expressed by the T cell and B7-1 (CD80) or B7-2 (CD86) expressed on the APC.

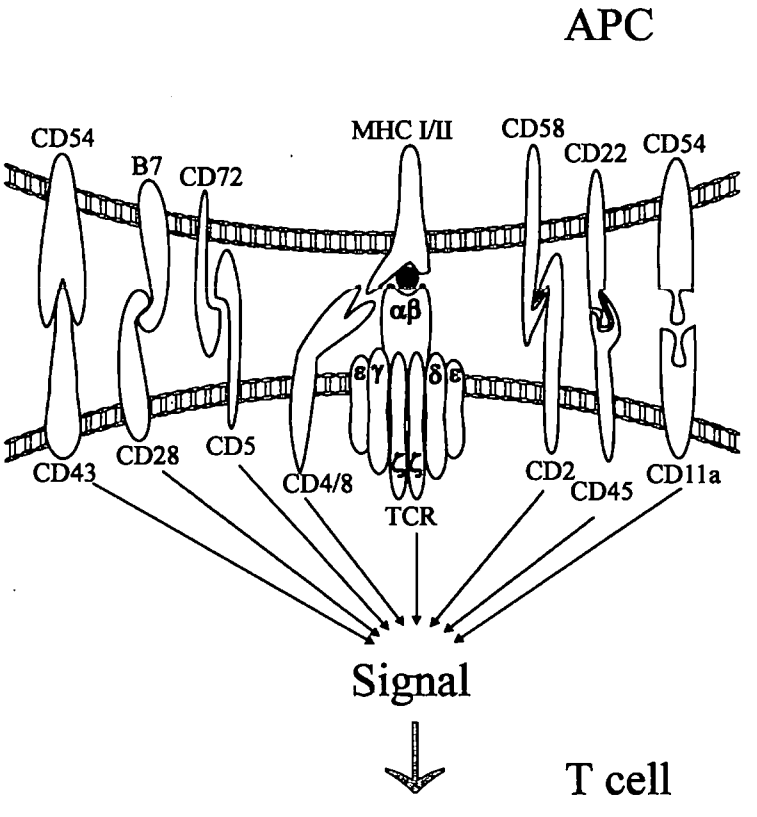

Figure 4. Molecules involved in TCR signal transduction (18).

Activation and signalling of the CD28 molecule induce an increase in the expression (43) and stability of the IL-2 mRNA (44). Furthermore, CD28/B7 ligation reduces the number of TCR/MHC-peptide interactions necessary for T-cell activation (45), and early events in CD28-mediated signal transduction lead to the activation of AP-1 and NFAT thereby integrating the TCR and CD28 activation signals (37). The CD28/B7 ligation therefore appears to be important, if not mandatory, for T-cell activation. However, other surface molecules do participate in the activation of T cells (Fig. 4) either by directly participating in signaling, leading to activation, by inducing sustained expression of e.g. B-7 (46), or simply by increasing the affinity of the cell to cell interaction (47). Additionally, it was recently shown that the induction of effector CTL (eCTL) is mediated by signaling through the CD40 molecule (48; 49).

The activation of a T cell after the encounter with an MHC/peptide complex may take one of several possible routes, meaning that the activation threshold can be gained by interactions between a number of different molecules, possibly differing from cell to cell and from antigen to antigen. The number and character of the different molecular interactions are therefore heavily influencing the consequences of TCR/MHC-peptide ligation. Possibly the differential fate of these interactions is, at least in part, mediated through quantitative differences in the phosphorylation of the ITAM of CD3 subunits (50; 51). That the peptide ligand is of utmost importance for the outcome of the TCR/MHC-peptide ligation is most clearly illustrated by the fact that agonistic peptides may, if altered at a single position, result in antagonistic TCR signaling and induce unresponsiveness (52).

TCR engagement with the MHC/peptide complex may therefore, depending on the antigen and the antigen quantity, the local environment and the state of the T cell result in activation, anergy or apoptosis.

Melanocytes and melanomas

Melanocytes originate from the neural crest and migrate to the skin during early embryonal life. Histologically, melanocytes are localised within the epidermal basal layer separated from each other by several keratinocytes (53). The prime function of melanocytes is the production of melanin, offering UV protection. Melanin synthesis starts with tyrosine as substrate and takes place in a special intracytoplasmic organelle, the melanosome. During melanin synthesis, the melanosome travels along the melanocytic dendrites, the end of which is eventually phagocytosed by the keratinocytes (54).

Frequently, melanomas arise from benign nevi, but also from normal skin. Although most cancers of the skin are non-melanoma skin cancers, melanoma is by far the most lethal. If not cured by surgery, melanoma is in most cases fatal.

Melanoma has been divided into four different classes based on growth characteristics and the confinement of growth to one or more tissue compartments (55). Class I encompasses precursor lesions (ordinary moles), class II the "melanoma *in situ*", class III and class IV are primary and metastatic melanoma, respectively. In addition, each class is divided into subgroups on the basis of growth direction (superficial vs. vertical growth), localisation and tumour type (lentigo maligna melanoma, LMM; superficial spreading melanoma, SSM; nodular melanoma, NM; or acral lentigenous melanoma, ALM) (55).

Several sequential steps of genetic changes describe tumour progression from melanocyte to melanoma, i.e. from normal cell to cancer cell (56). Chromosomal abnormalities in melanoma involve chromosomes 1p, 6q, 7q, 9p, 10q, 11q, 22, and Y (57; 58). Some of the genetic changes harboured in malignant melanoma have been characterised in more detail, the most frequent being deletions or mutations of the p16 (59), PTEN (60), and beta-catenin genes (61). Furthermore, other less frequent genetic changes have been described (62; 63) indicating that tumour initiation and promotion may take one of several pathways.

Melanoma immunology

The past decade has unveiled new insights into the role of T lymphocytes in the host's immune response to cancer in general and to melanoma in particular (64). Several melanoma associated antigens (MAA) recognised by T cells have been characterised, and a number of HLA class I and class II restricted peptides have been identified (65). These antigens can be divided into three different groups: tumor-associated testis specific antigens, melanocyte differentiation antigens, and mutated or aberrantly expressed antigens (Table 2). Several of these proteins give rise to more than one antigenic peptide; the number of known melanoma associated peptides that can induce killing by CTL has exceeded thirty and is still increasing. Furthermore, most studies concerned with the isolation and characterisation of melanoma associated proteins have been carried out using melanoma cells expressing the HLA-A1 or the HLA-A2.1 allele. Therefore, even if no additional melanoma associated proteins will be discovered, the antigenic peptides derived from the known proteins will increase as more restriction elements are studied. Some of the peptides have only been shown to induce cytotoxicity against peptide loaded cells, and it is not known whether these peptides are actually processed and presented naturally. Nevertheless, it is evident that melanoma cells do express a number of proteins that give rise to antigenic peptides.

In line with these findings, clinical data indicate that the immune system is essential in the control of tumor growth. A brisk infiltration of lymphocytes is associated with a favourable prognosis (55) and complete or partial regression of primary melanoma occurs quite frequently (66). Cytotoxic T-cell lines and clones which recognise autologous melanoma cells in an HLA restricted manner have been established in several laboratories (67-69), and melanoma specific cytotoxic T-cell precursors have been detected in the blood of melanoma patients (70-72).

Table 2. Antigenic epitopes of melanoma-associated antigens recognized by CTL (Kirkin et al., 1998).

Antigen	HLA class I	Epitope	Sequence	References
Cancer/testis-specific antigens				
MAGE-A1	A1	161-169	EADPTGHSY	(73)
	Cw16	230-238	SAYGEPRKL	(74)
MAGE-A2	A2	112-120[a)]	KMVELVHFL	(75)
	A2	157-166[a)]	YLQLVFGIEV	(75)
MAGE-A3	A1	168-176	EVDPIGHLY	(76)
	A2	271-279[a)]	FLWGPRALV	(77)
	A24	195-203[a)]	IMPKAGLLI	(78)
	B44	167-176[a)]	MEVDPIGHLY	(79)
BAGE	Cw16	2-10	AARAVFLAL	(80)
GAGE-1	Cw6	9-16	YRPRPRRY	(77)
PRAME	A24	301-309	LYVDSLFFL	(81)
NY-ESO-1	A2	157-165	SLLMWITQC	(82)
	A2	157-167	SLLMWITQCFL	(82)
	A2	155-163	QLSLLMWIT	(82)
Melanocyte differentiation antigens				
gp100	A2	154-162	KTWGQYWQV	(83)
	A2	209-217	ITDQVPFSV	(83)
	A2	280-288	YLEPGPVTA	(83)
	A2	457-466	LLDGTATLRL	(84)
	A2	476-485	VLYRYGSFSV	(83)
	A3	17-25	ALLAVGATK	(85)
MART-1	A2	27-35	AAGIGILTV	(86)
	A2	26-35	EAAGIGILTV	(87)
	A2	32-40	ILTVILGVL	(88)
Tyrosinase	A1	243-251	KCDICTDEY	(89)
	A2	1-9	MLLAVLYCL	(90)
	A2	369-377	YMNGTMSQV	(90)
			YMDGTMSQV[b)]	(91)
	A24	206-214	AFLPWHRLF	(92)
	B44	192-200	SEIWRDIDF	(93)
TRP-1/gp75	A31	1-9[c)]	MSLQRQFLR[c)]	(94)
TRP-2	A31,A33	197-205	LLPGGRPYR	(95)
Mutated and aberrantly expressed antigens				
MUM-1[d)]	B44	nt782-808	EEKLIVVLF	(96)
β-Catenin	A24	29-37	SYLDSGIHF	(97)
p15	A24		AYGLDFYIL	(98)
CDK4	A2		ACDPHSGHFV	(99)
GnT-V[e)]	A2		VLPDVFIRC(V)	(100)
gp100-in4	A24	170-178	VYFFLPDHL	(101)

[a)] Predicted epitopes, to which induced CTL can kill tumor cells
[b)] Natural peptide identified by elution from HLA-A2
[c)] Antigenic peptide resulted from translation of an alternative open reading frame (ORF3) of the gp75 gene
[d)] Mutation occurs in an intron sequence introducing a stop codon.
[e)] N-acetylglucosaminenyltransferase V. The peptide is encoded by an intron sequence.

Several studies have focused on the characterisation of the T-cell response against malignant melanoma *in situ*, and evidence has been provided for the presence of clonally expanded T cells in both primary and metastatic lesions (102-108). These data strongly indicate that melanoma cells present antigenic peptides in the context of HLA molecules, and that T cells capable of recognising these antigens are detectable *in situ*.

However, the response is obviously inadequate to control tumor growth, as tumour progression often occurs, raising the question of how melanoma cells escape immune surveillance. One frequently suggested mechanism is that tumor cells lose the expression of MHC class I molecules, rendering them invisible to the T cell. Most studies demonstrating loss of expression of class I molecules in melanoma have been carried out using histological staining of melanoma lesions. This approach is hampered by the low sensitivity of this technique – at least 200 molecules are required for a positive stain and large areas of the tumours are often necrotic. Furthermore, in contrast to these findings, very few class I negative melanoma cell lines have been described (109). Therefore other mechanisms are more likely to cause the immune escape of melanoma.

It has been suggested that TILs in melanoma and even peripheral T cells from melanoma patients have an impaired signaling capacity through the TCR/CD3 complex due to downregulation of the CD3 ζ-chain (110). The mechanism underlying this downregulation remains elusive.

Melanoma cells are known to secrete a number of different cytokines some of which may suppress cellular immune responses e.g. IL-10 (111; 112) and TGFβ (113; 114). Normal melanocytes are responsive to growth inhibition to TGFβ, whereas melanomas are not. In other tumour types unresponsiveness to TGFβ have been shown to be accomplished by mutations in Smad4/DPC4, a gene encoding a protein essential to TGFβ mediated intracellular signaling (115-117). However, in the analysis of more than 40 melanoma cell lines we detected no Smad4/DPC4 mutations (thor Straten & Guldberg, unpublished data). It is possible that unresponsiveness to TGFβ in melanoma is achieved by deletions or mutations of p15.

Another immune escape mechanism likely to be used by melanoma cells is the expression of the Fas ligand, implying that T cells that attempt to encounter the melanoma cells may be killed if they are susceptible to Fas mediated apoptosis (118; 119).

A number of immunotherapeutic trials have been carried out with the aim of boosting the (ongoing) T-cell response. Earlier trials were in most cases conducted by the systemic administration of immunomodulatory cytokines such as IL-2 (120). Although some beneficial effects were observed, the general results were not encouraging, as the response rates were limited and the side effects severe

(121). One of the major obstacles is that the systemic administration of cytokines neglects the paracrine nature of cytokines. This limitation has been overcome in a murine tumor model by the use of targeted IL-2 therapy using a fusion protein consisting of a tumor-specific antibody and IL-2 (122; 123). Using targeted IL-2 therapy it has been shown that established metastases could be eradicated in a response mediated by CD8 T cells (122). Furthermore, it was recently shown that tumor eradication is presumably accomplished by the activation of T cell clones present in the tumor lesion prior to therapy (124). However, this therapeutic model has not yet been tested in humans. Another approach, which has been used in a clinical setting, is the adoptive transfer of autologous *in vitro* expanded lymphokine activated killer (LAK) cells or TIL; either alone or in combination with IL-2 (125). The objective responses have been limited.

Discovery of tumor regression antigens and the resulting peptides has prompted peptide based vaccination trials. Immunisation of HLA-A2 positive melanoma patients have been carried out using subcutaneous injections of peptides derived from MART-1, gp100 and tyrosinase. However, no tumor regressions were observed (126). Likewise the subcutaneous injection of the MAGE-3/HLA-A1 peptide only revealed minor responses (127). Very recently, two different vaccination trials were conducted using DCs pulsed with tumor lysates or peptides. The results were impressive showing partial or complete regression in more than 40% of the patients (128; 129). Whether these promising results will stand the test of more extensive randomised trials remains to be established.

Scientific Results

Aims of the study

The primary aim of the present study has been to investigate the role of T cells infiltrating human melanoma, with specific focus on the analysis of *in situ* T cells. Most studies of TIL in melanoma have been concerned with melanoma cell lines and *in vitro* propagated TIL. These investigations have revealed important new insight into the MAA expressed by melanoma cells and the T-cell recognition of these, as summarised on the preceding pages. These *in vitro* findings have made it plain that the biological foundation in terms of a T-cell response against melanoma was not unsound. However, it also made clear that the *in vitro* situation was markedly different from the *in vivo* situation. In contrast to the *in vitro* cytotoxicity data against well-characterised MAA, the *in vivo* T-cell response is obviously insufficient to clear tumour burden. To get a glimpse of what goes on *in situ*, we have aimed at investigating melanoma lesions for the presence of clonotypic T cells.

The present Thesis is introduced with a discussion of reverse transcription polymerase chain reaction (RT-PCR) based analysis of TCRBV regions, including the description of a panel of primers. Using this primer panel, we analysed for TCR clonality in progressive *versus* regressive parts of partially regressive primary melanoma lesions (paper 1). The main conclusion drawn from this study is that clonotypic T cells were present in both regressive and progressive parts of the same lesions. However, sequencing of "non clonal" BV-regions demonstrated that most of them were not polyclonal by nature but oligoclonal e.g. that in some cases all transcripts were of the same length or only a limited number of BJ regions were used. This indicated that some T-cell clones were left undetected by direct sequencing.

To facilitate full and detailed analysis of T-cell clonotypes in the infiltrates we aimed at establishing a suitable method for the detection of clonally expanded T cells. Denaturing gradient gel electrophoresis (DGGE) was chosen because this method is highly sensitive and excludes laborious steps that are obligatory in almost all other methods dealing with the detection of TCR clonality (paper 2).

We applied the DGGE based method to analyse for *in situ* T-cell clonality in 6 subcutaneous melanoma lesions from two patients. The results demonstrated two important characteristics of *in situ* TIL in melanoma. First, the infiltrate constitutes an exceedingly high number of different T-cell clones, and second, the T-cell response appears to be executed by local T cells that do not enter the periphery (paper 3).

The final study deals with a comparative analysis of *in situ* T cells and T cells propagated *in vitro*, demonstrating that standard *in vitro* culture conditions do not support the growth of *in vivo* expanded T-cell clones (paper 4).

RT-PCR based analysis of T-cell receptor variable regions β1-24

Abstract

The present report describes a series of oligonucleotide primers specific for TCR variable regions. The primer panel consists of 5′ primers covering the TCRBV regions 1-24 and a 3′primer matching to the TCRBC regions. Primers were selected for best possible match to the relevant TCRBV families and a minimum of 5 mismatches to all other families.

By using the computer program Oligo 3.4, primers were chosen to produce PCR products within a limited size range, to have a minimum of internal secondary structures and primer dimers, to have similar melting temperatures (T_m) and ΔG values, and to work using standardised conditions. The specificity and efficiency of the primer panel were tested in reverse transcription polymerase chain reaction (RT-PCR).

Introduction

The polymerase chain reaction has been the method of choice in most laboratories aiming at analysing for the expression of TCRBV regions. Although several different PCR based methods for that purpose have been described, the most commonly used has been reverse transcription polymerase chain reaction (RT-PCR), in which TCR transcripts is amplified with a set of primers covering the variable region families, together with a common constant region primer.

For the purpose of analysing T-cell infiltrates in malignant melanoma, RT-PCR was chosen because of its capacity to analyse minute amounts of material and its ability to produce semiquantitative data. Furthermore, using PCR based methods it is possible to proceed directly through steps of cloning or sequencing. However, the validity of semiquantitative RT-PCR is highly dependent on parameters such as primer stability in duplex formation, specificity and discrimination of non-targets. Furthermore, it is imperative that all reactions work well and are being carried out within the linear range of amplification.

Materials and Methods.

Selection of primer sequences

The primers for amplification of BV regions 1-24 were carefully selected with the aim of perfect match to the BV family in question and all sub-members of the family. Sequences of TCRBV regions were obtained from the GenBank Database using the accession numbers given by Arden et al. (23). Sequences not available in the GenBank Database were typed in by hand. Library files were created for each TCRBV family using the PC/GENE "FILE" program (Intelligenetics, Palo Alto, CA, USA). Using the PC/GENE program "CLUSTAL", the sequences in each family were aligned, and primer sequences were found in regions of optimal consensus. Optimal primer sequences were found using the computer program Oligo Version 3.4 (Medprobe, Oslo, Norway) (130) aiming at ΔG below -40.0 and a T_m between 50°C - 60°C. The BV primers BV1, BV10, BV11 and BV22 have been described (131). All primers were subsequently tested for potential homology to all other families, aiming at a minimum of 5 mismatches to non-relevant BV templates. Most of the primers used in this study meet these criteria although in some families it was not possible to achieve 100% match to all subfamily members using a single primer. For that reason some BV families are covered by two primers (BV12 and BV13), or by the use of mixed base positions in the primer (BV5 and BV6). In other instances mismatches to some BV subfamily members were accepted in the 5′-end of the primer. The sequences of the primers, numbered according to Arden et al. (23), are given in Table 3.

Table 3 Oligonucleotide primers for the human TCRBV families.

BV	Sequence	Pos	Size	ΔG	T_m
BV1	5'-CCGCACAACAGTTCCCTGACTTGC-3'	-92	456	-47.5	63.5
BV2	5'-TCTCATGCTGATGGCAACTTCCAAT-3'	-163	527	-46.5	60.6
BV3	5'-CGCTTCTCCCTGATTCTGGAGTCC-3'	-66	430	-47.1	61.2
BV4	5'-TTCCCATCAGCCGCCCAAACCTA-3'	-92	456	-49.9	65.0
BV5	5'-CTGAG C/$_A$TGAATGTGA A/$_G$C G/$_A$CCTTG-3'	-60	424	-42.0	56.0
BV6	5'-MAGRATGTARMKCTCAGGTGTGAT-3'	-135	582	-40.0	50.0
BV7	5'-TAAGAAGTCTTTGAAATGTGAACAAC-3'	-221	585	-47.2	62.0
BV8	5'-TGAAGATCCAGCCCTCAGAACCC-3'	-56	420	-45.3	60.2
BV9	5'-AACAGGACTCTAAGAAATTTCTGAAG-3'	-175	539	-42.5	49.4
BV10	5'-CCACGGAGTCAGGGGACACAGCAC-3'	-43	407	-47.8	65.5
BV11	5'-TGCCAGGCCCTCACATACCTCTCA-3'	-43	407	-47.4	63.5
BV12	5'-GAGAATTTCCTCCTCACTCTGG-3'	-70	434	-39.2	51.2
	5'-GACCTCCCCCTCACTCTGG-3'	-70	434	-37.8	53.0
BV13	5'-CACTGAGATGTACCCAGGATATGA-3'	-220	584	-40.2	52.4
	5'-TGCAGTGTGCCCAGGATATGA-3'	-217	581	-39.8	56.2
BV14	5'-GGGCTGGGCTTAAGGCAGATCTAC-3'	-165	529	-48.2	60.3
BV15	5'-CAGGCACAGGCTAAATTCTCC-3'	-79	443	-40.2	53.1
BV16	5'-GATGAGTCCGGTATGCCCAACAATC-3'	-117	481	-47.8	61.3
BV17	5'-CCCCAAAGTACCTGTTCAGAAA-3'	-263	627	-40.4	52.6
BV18	5'-TTTCTGCTGAATTTCCCAAAGAGG-3'	-91	455	-45.7	57.7
BV19	5'-TCTCAATGCCCCAAGAACGCAC-3'	-87	451	-44.5	60.8
BV20	5'-AGGTGCCCCAGAATCTCTCAG-3'	-105	469	-40.3	54.7
BV21	5'-GAYGATTCACAGTTGCCTAAGGA-3'	-119	483	-41.5	53.8
BV22	5'-AAGTGATCTTGCGCTGTGTCCCCA-3'	-234	598	-47.4	63.5
BV23	5'-GCAGGGTCCAGGTCAGGACCCCCA-3'	-172	536	-42.4	60.3
BV24	5'-ATCCAGGAGGCCGAACACTTC-3'	-87	451	-42.0	56.8
BCseq2	5'-ACAGCGACCTCGGGTGGGAA-3'	97		-43.4	61.2
BC4	5'-CGTAGAACTGGACTTGACAGCGG-3'	304		-43.3	57.7
BC-5'	5'-TGAGCTGGTGGGTGAATGGG-3'	187	343	-40.5	57.9
BC-3'	5'-GGTGGCCTTCCCTAGCAGG-3'	530		-40.7	55.9

Position +1 is defined as the first nucleotide after the sequence coding for the conserved amino acid sequence CASS in the proximal end of the variable domain. The approximate sizes of the PCR products are calculated using an estimated mean length of the DJ region of 60 bp.

Isolation of RNA and synthesis of cDNA

RNA was extracted by a modification of the method described by Chomczynski and Sacchi (67; 132). cDNA synthesis was performed according to the manufacturer's protocol using M-MLV reverse transcriptase (Life Technologies Inc., Gaithersburg, MD, USA) primed with oligo-dT. Incubations were performed at 42°C for 30 min, 99°C for 5 min. and frozen or used immediately.

RT-PCR conditions and parameters

Several different PCR parameters were tested with specific focus on annealing temperature (55°C, 58°C, 60°C, 62°C and 65°C). Several primers gave rise to spurious amplification products at an annealing temperature of 55°C and 58°C, and some primers were inefficient at an annealing temperature of 62°C (data not shown). At 60°C, most primers worked well although some unspecific amplifications were identified. To further increase specificity, "touch down" (133) and "hot start" (134) cycling parameters were introduced. These stratagems, in particular the "hot start", increased specificity while leaving efficiency unaffected (data not shown). The parameters used for amplification were 94°C for 30 sec, 60°C for 30 sec. and 72°C for 60 sec., using "touch down" going from 65°C to 60°C over the first 10 cycles. Taq polymerase and dNTPs were added to the reaction tube at an 80°C step between the denaturation and annealing steps of the first cycle.

In addition to annealing temperature other reaction conditions affecting the specificity and sensitivity were tested. These experiments included changes in primer and nucleotide concentrations, Taq polymerase from different manufacturers and different concentrations of enzyme. Eventually, amplifications were carried out in 50mM KCl, 20 mM Tris pH 8.4, 2.0 mM $MgCl_2$, 0.2 mM cresol red, 12% sucrose, 0.005% (w/v) BSA (Boehringer-Mannheim, Mannheim, Germany)) using 2.5 pmol of each primer, 40 μM dNTPs (Pharmacia LKB, Uppsala, Sweden) and 1.25 units of AmpliTaq polymerase (Perkin Elmer Cetus Corporation, Emeryville, CA.). The inclusion of cresol red and sucrose in the reaction buffer enables direct loading to the gel (135).

Semiquantitative RT-PCR

For quantitative analysis either Southern blot analysis was carried out as described (136), or the BC primer was labeled with ^{33}P, aliqouts electrophoresed in an agarose gel, and the gel dried prior to exposure to Molecular Dynamics Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA, USA). In either case quantitation was accomplished using the Imagequant software (137).

To validate the semiquantitative RT-PCR methodology, serially diluted cDNA was PCR amplified for different numbers of cycles (26, 28, 30, 32, and 34) using the primers BV 1-24 together

with the constant region primer BC4. Data from these experiments were used to determine the amount of cDNA and the number of cycles through which the specific PCR products accumulated exponentially, enabling determination of the relative abundance of each TCRBV region. Furthermore, the accuracy and reproducibility of the method were investigated by repeated analyses of peripheral blood lymphocytes (PBL) indicating that the experimental variation represented 4-8% of the mean value. The primers BC-5′ and BC-3′, matching to TCRBC exons 1 and 3 respectively, were used to amplify all TCR cDNAs in each sample. The amount of TCRBC PCR product was quantitated using Imagequant software (137), ensuring that all TCRBV analyses were carried out using equal amounts of TCR cDNA.

Results and Discussion

Since the PCR methodology was first described in 1985 (138) and later in 1987 using a thermostable Taq polymerase (139) it has become one of the most widely used techniques in scientific research. Initially, PCR was not viewed as a quantitative method, but the ability to analyse small amounts of nucleic acids thereby enabling quantitative analysis to be carried out in situations where RNA amounts were too small for conventional Northern blotting, were too obvious to be overlooked. For the analysis of multigene families such as the TCR, the field opened with the paper of Choi et al. in 1989 (140) and since then quantitative PCR of TCR variable regions have been conducted in many laboratories.

A major obstacle in semiquantitative RT-PCR based analyses of multigene families is to select primers that work equally well and do not crossreact within the different families. Since the first primer panels for TCR α/β chains were published almost a decade ago, the full genomic DNA sequences of TCRAB regions have been published (24; 25), and the classification of different BV/AV sequences into the relevant families has recently been updated (23). Obviously, primer panels for the amplification of multigene families must be updated according to available sequence data in order to ensure that all members of the different families are amplified. For this purpose, the publication of all TCRV sequences has greatly aided the selection of specific primers. The vast majority of the previously published primer panels either do not match optimally to all sub-members of the different families, or show potential crossreactivity to other families (140-146). Actually, with regards to crossreactivity only a single primer panel has been published for which this topic has been included in the selection of the primer sequences, although with the more recent updates of sequence data not all primers match optimally to the target sequences (131).

In the present study, TCRBV sequences were put into library files according to the BV family and aligned to find sequence stretches of optimal consensus in which primer sequences could be selected. All primer sequences were tested for match to non-relevant BV families aiming at a minimum of 5 mismatches. Most importantly, however, primers were tested for the ability to amplify the BV region in question with high efficiency. During the optimisation steps of the PCR parameters and conditions, several primers were changed in order to have a panel of primers that worked equally well,

with high sensitivity, stability and efficiency. Fig. 5 depicts RT-PCR products of TCRBV regions 1-24 electrophoresed in agarose and stained with ethidium bromide (EtBr).

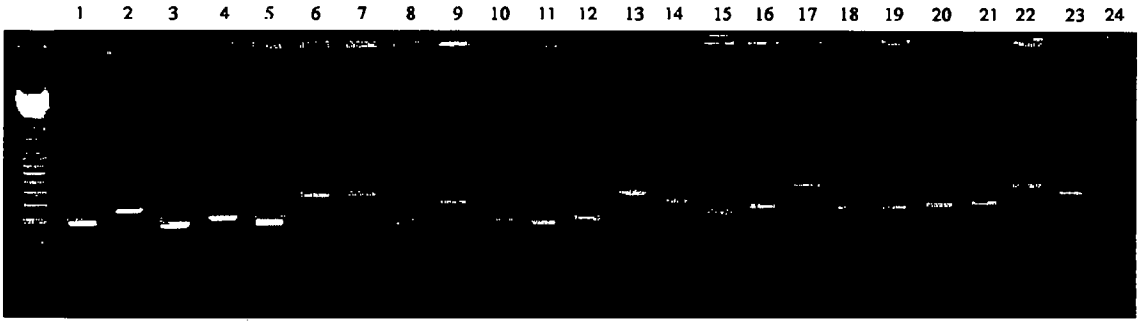

Figure 5. RT-PCR amplification products of TCRBV1-24 transcripts in peripheral blood lymphocytes of healthy donors. Visualised by staining with EtBr.

For semiquantitative analysis using RT-PCR many researchers take advantage of co-amplification of a control TCR α-constant chain PCR product in each tube, in order to equalise for any differences in the amount of target or other tube to tube differences. In the present study, several experiments were conducted, demonstrating that the amplification efficiency of two different targets in the same tube did not always reflect each other. In other words, the efficient amplification of one of the targets in the tube does not necessarily imply that the other target is amplified equally efficiently (147). Furthermore, it became clear that "duplex" amplifications in many situations decrease the amplification efficiency of both targets and increase the risk of spurious amplification products. Consequently, semiquantitative analysis of TCRBV regions in the present study has been carried out without co-amplification. All reactions are prepared as master reaction mixtures containing all reagents except the BV primer, and master mixture aliquoting is being conducted with high precision.

Nevertheless, semiquantitative PCR has its limitations. Preliminary experiments were conducted to show that the amount of cDNA used would amplify all 24 TCRBV regions exponentially when PBL cDNA was amplified. In general the probable error represented 4-8% of the mean value in duplicate experiments. However, the experimental variation may reach higher values both at very low and at very high concentrations of target. At very low signal strengths the background values may constitute a relatively high proportion of the signal, even with highly sensible detection systems such as

the Phosphor Imager. This means that small changes in the background values over the gel, and from one gel to another, may induce significant differences even when the same sample is run in different gels. It is therefore not possible to make valid distinctions between different values below 2%.

At very high target concentrations the increased error rate is related to the nature of PCR. When a single BV region constitutes close to the majority of the T-cell population, the present setup will not ensure that all reactions stay within the exponential phase of the reaction. For that reason, the PCR amplification of TCR transcripts expressed at extremely high levels, will reach the "plateau phase" and consequently be underestimated with regards to the level of expression. In PBL cDNA the relative abundance of the different BV regions is normally in the range of 2-12 %, meaning that none of the TCRBV regions dominates the pool of TCR messengers. This may not be the situation when different T-cell infiltrates are analysed in which single BV regions may constitute the majority of the infiltrate (136). However, with the relation between the number of cycles and the amount of cDNA used in the present study, reasonable estimation of BV region expression can be made even when single BV regions constitute up to approximately 30% of the TCR messenger pool (136).

These considerations clearly illustrates that any setup of semiquantitative RT-PCR based analysis of multigene families necessarily will be a compromise of being able to detect and quantitate targets expressed at very different levels. Possibly one of the major pitfalls in semiquantitative PCR therefore is related to the understanding of these limitations in the evaluation of the data.

Clonal T-cell responses in tumour infiltrating lymphocytes from regressive and progressive regions of primary human melanoma

Abstract

The TCRBV region repertoires of TIL from progressive and regressive parts of the same primary human melanoma lesions were characterised by RT-PCR. After surgery, the tumours were divided into different parts that were judged as regressive or progressive regions by visual inspection. Subsequently this diagnosis was confirmed by histology. From a total of 4 primary melanomas analysed, 2 were shown to be HLA-$A2^+$.

Only relatively few BV-region families were expressed at significant levels in each of the samples. Comparison of the BV-region expression in regressive versus progressive regions of the same tumour revealed major differences in all cases examined. Direct sequencing of RT-PCR products indicated that highly expressed BV-regions of clonal origin were present in both regressive and progressive regions.

Together, these data strongly suggest the occurrence of clonal T-cell responses in both regressive and progressive areas of the same primary tumour. The differences in expression of certain BV-regions may correlate with the functional activity of certain populations of tumour-infiltrating T cells.

Introduction

The prognosis of melanoma, which cannot be cured by surgical resection, is one of the most unfavourable in medicine. The coexistence of tumour specific immunity with a progressing tumour remains a major paradox of tumour immunology. This enigma is most evident in partially regressing melanoma, where efficient eradication of tumour cells occurs in close vicinity to uncontrolled tumour growth. Everson and Cole (148) have defined spontaneous regression of malignant melanoma as the partial or complete disappearance of a malignant tumour in the absence of all treatment. Such spontaneous regressions of primary cutaneous melanoma are not uncommon and are clinically manifested by a decrease in the amount of pigmentation, variation in colour, scarring and division of the original lesion into multiple smaller lesions.

The usage of TCRBV region repertoires of TIL in primary human melanomas has recently been characterised indicating the presence of clonally expanded T cells in the tumour (102; 149; 150). However, all studies thus far have analysed the TCR V-region repertoire in the total tumour sample. The occurrence of spontaneous regression of primary melanoma is correlated with a high degree of lymphocyte infiltration (55). Therefore, it is possible that clonal T-cell responses may originate exclusively from the regressive parts of the tumour.

To test this notion, TCRBV region repertoires in regressive versus progressive regions of different primary melanomas were analysed by RT-PCR. Only very few BV-gene families were expressed at high levels with major differences in regressive *versus* progressive regions of the same tumour. Highly expressed BV-regions of clonal origin were found in both regressive and progressive parts of the same tumour, suggesting a clonal T-cell response to occur in both parts of malignant melanoma primary tumours.

Materials and Methods

Tumour samples

Fresh primary melanoma lesions were received as biopsy material immediately after surgery performed at the Department of Dermatology, School of Medicine, Würzburg. Four patients were included in this study. The tumours were examined for Clark level, type, localisation and thickness. The tumours were separated into progressive and regressive regions by visual inspection that was subsequently validated by histological analysis (151).

Isolation of RNA and synthesis of cDNA

RNA was extracted by a modification of the method described by Chomczynski and Sacchi (67; 132). cDNA synthesis was performed according to the manufacturers' protocol using M-MLV reverse transcriptase (Life Technologies Inc., Gaithersburg, MD, USA). Incubations were performed at 42°C for 30 min, 99°C for 5 min. and frozen or used immediately.

In order to analyse equal amounts of TCR cDNA from each sample, different cDNA dilutions were amplified by PCR with primers for the constant region of the B-chain (Primers BC-5′ and BC-3′ 1. PCR amplification; 20 cycles, reamplification; 35 cycles). The amounts of cDNA to be used for the BV-analyses was determined on the basis of the intensity of these PCR-products observed after agarose gel electrophoresis and subsequent staining with EtBr.

TCRBV PCR

In the present study we analysed for the expression of TCRBV regions using 24 primers covering the BV regions 1-24 in conjunction with a common BC primer (136). Since the amount of cDNA was limited, each analysis was performed initially with 20 cycles using BC4 as 3′-primer. Aliquots from this first amplification were diluted 1/100 and 5 µl of this sample were amplified by further 35 cycles of PCR using a nested 3′-primer (BCseq2, Table 3). Reactions were carried out as described (136), with the exception that in the reamplification reaction, the primer concentration was raised from 1,25 to 5 pmol and the dNTP concentration was raised from 10 to 40 µM.

Cross contamination was avoided by use of plugged pipette tips (Mμlti Technology Inc. Salt Lake City, Utah, USA) and separate rooms for pre- and post-PCR handling. Negative controls were samples without cDNA. All amplifications were performed in a Hybaid Omnigene Thermal Cycler (IIybaid, Teddington, Middlesex, UK) using standard microtubes (Mμlti Technology Inc., Salt Lake City, Utah, USA). For analysis, 15 μl aliquots of PCR products were subjected to electrophoresis in a 3% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME) for 3 hours at 100 V.

HLA-A2 PCR

Analysis of HLA-$A2^+$ mRNA expression was performed by RT-PCR. The parameters were as described above with the exception that the "touch down" were carried out from 70°C to 62°C. Two different HLA-$A2^-$ samples were analysed as negative controls. Primers used were 5′-GGGACGGG GAGACACGGAAA-3′ (sense) and (5′-AGCGGATCCAGTCATATGCGTTTTGGGGGC-3′ (antisense) taken from published HLA-A2 cDNA nucleotide sequence (152). The underlined sequence corresponds to an added Bam-H1 site. The resulting PCR product consists of 390 bp.

Southern Blots

After electrophoresis, PCR products were transferred to nylon membranes and hybridised as previously described (136). 1/1 mixtures of P^{33} labelled oligonucleotides, BHYB1 (5'-AACACCTTGTTCAGGTC-3') and BHYB2 (5'-AACACGTTTTT CAGGTC-3′), which match BC1 and BC2, respectively, were used as probes. After washing, the radioactive probes were visualised using a Molecular Dynamics Storage Phosphor Phosphoimager (137).

Sequence analysis

Highly expressed BV-regions were sequenced in order to investigate the clonality of their expression. After re-amplification, the PCR products were extracted from agarose gel, precipitated with ethanol and resuspended in 10 μl 1XPCR buffer (Perkin Elmer Cetus, Emeryville, CA). Sequenase version 2.0 (USB Corporation, Cleveland, OH, USA) was used following the manufacturers' suggestions.

However, in order to sequence directly, we used a ^{33}P labelled primer (Cβseq2) and denaturation was carried out by boiling for 5 min in a total volume of 12 µl. The gels were analysed with a Molecular Dynamics Phosphoimager.

Results

Histopathology

The histopathological characteristics of the tumour samples are given in Table 4.

Table 4. HLA-A2 expression and histopathological characteristics of the tumour samples.

Tumour	HLA-A2	Localisation	Type*	Thickness	Clark level
1	-	Trunk	NMM	1,5mm	V
2	+	Arm	SSM	1,4mm	IV
3	-	Leg	SSM	1,8mm	IV
4	+	Trunk	SSM	1,9mm	IV

*SSM: superficial spreading melanoma; NM: nodular melanoma

TCRBV 1-24 expression

RT-PCR was used to compare the TCRBV repertoire of TIL infiltrating regressive and progressive parts of 4 primary melanoma tumours. Each amplified RT-PCR product was obtained with the expected size as a single band in all cases. A limited number of BV-regions were expressed at significant levels in each sample. The number of different BV-regions expressed at significant levels in each

Table 5. TCRBV expression and clonality in the tumour samples.

Tumour 1 progressive	BV4*, BV5*, BV8‡, BV13*
Tumour 1 regressive	BV3*, BV5, BV13*, BV18
Tumour 2 progressive	BV4*, BV12, BV14
Tumour 2 regressive	BV2*, BV4, BV12, BV20*
Tumour 3 progressive	BV13*, BV14*, BV20
Tumour 3 regressive	BV6*, BV13, BV21‡, BV24*
Tumour 4 progressive	BV3, BV5, BV14*, BV15‡, BV17‡
Tumour 4 regressive	BV1*, BV3‡, BV5, BV7, BV13‡, BV15,

TCRBV-regions expressed in progressive and regressive parts of the tumour samples. *, regions shown by sequence analysis to be of clonal origin. ‡, regions not sequenced.

tumour ranged from 7-11. In general, the regions BV3, BV4, BV5, BV13, BV14 and BV20 were expressed in more than one tumour, whereas the regions BV1, BV2, BV6, BV7, BV8, BV12, BV15, BV17, BV18, BV21 and BV24 were expressed in one tumour only (Table 5).

In no instance had the regressive/progressive parts of the same tumour a similar pattern of BV gene expression. For example, both patients 1 and 4 show expression of BV3, BV5 and BV13. However, regions BV1, BV4, BV7, BV14, BV15 and BV17 are expressed exclusively in one of the tumours. Some BV regions were not expressed in any of the tumours, including BV9, BV10, BV11, BV16, BV19, BV22 and BV23. Comparing the expression of specific TCRBV in the different areas of the tumour in all cases showed major differences. Examples of this phenomenon are the expression of BV18 which is highly expressed in sample 1 progressive (prog.) and absent in sample 1 regressive (reg.); BV1 in tumour 4 reg. but absent in tumour 4 prog. and BV14 present in tumour 4 prog. but not in tumour 4 reg. (Fig 6), and BV20, which is highly expressed in tumour 2 reg. but absent in tumour 2 prog. However, in all tumours analysed one or a few regions were expressed in both parts of the tumour (Table 5)

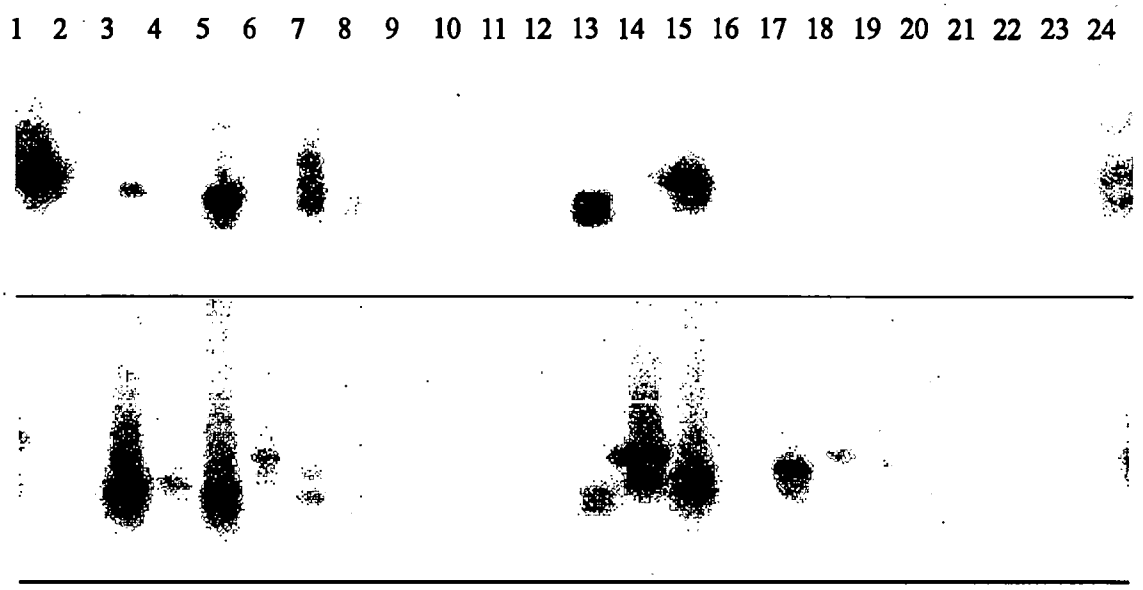

Figure 6. Southern Blot analysis of PCR amplification products of TCRBV regions 1-24 in biopsy material from tumour 4; upper, regressive part; lower, progressive part.

HLA-A2 RT-PCR

The expression of HLA-A2 was examined with specific primers for HLA-A2 and RT-PCR under highly stringent conditions. The results showed correctly sized PCR products derived from the amplification of cDNA from tumours 2 and 4 (Table 4).

Sequence analysis

Several BV-regions were sequenced in order to investigate whether any of these regions were of clonal origin. From a total of 28 PCR products sequenced, 14 of these revealed distinct CDR3 regions, all showing the expected BV-region sequence (Table 6). Readable CDR3 sequences were found in both regressive and progressive parts of the same tumours. In the samples 1 reg./1 prog., both the regressive and the progressive regions of this tumour showed expression of BV13. The sequence analysis in both cases showed a readable CDR3 region; however the sequences were different. In tumour 4, both parts of the tumour showed a readable CDR3 region (BV14 and BV1, respectively). However, the BV5 region expressed in both parts of this tumour showed a non-readable CDR3 region. Table 6 depicts the nucleotide sequence as well as the deduced amino acid sequence from those sequences showing a readable CDR3.

Table 6.

Tumour		TCRBV / CDR3 / Joining region
Tumour 1 prog.	BV4J2S3	TAT CTC TGC AGC GTT GGG TTA CTA GGG ACA CAG TCC ACA GAT ACG CAG TAT TTT GGC CCA GGC ACC CGG CT Tyr Leu Cys Ser Val Gly Leu Leu Gly Thr Gln Ser Thr Asp Thr Gln Tyr Phe Gly Pro Gly Thr Arg Leu
	BV5J2S5	TAT CTT TGC GCC AGC ACC TGG GGC GCG GGG ATA GAG ACC CAA ACC TTC GGG CCA GGC ACG CGG CTC CTG GTG CT Tyr Leu Cys Ala Ser Thr Tpr Gly Ala Gly Ile Glu Thr Gln Thr Phe Gly Pro Gly Thr Arg Leu Leu Val
	BV13J2S2	TAC TTC TGT GCC AGC AGG GCC GGG GGC CCG AAC ACC GGG GAT CTG TTT TTT GGA GAA GGC TCT A Tyr Phe Cys Ala Ser Arg Ala Gly Gly Pro Asn Thr Gly Asp Leu Phe Gly Glu Gly Ser Arg
Tumour 1 reg.	BV3J1S1	TAC CTC TGT GCC AGC AGT TTA CCG GAC GAG AGG AAC ACT GAA GCT TTC TTT GGA CAA GGC ACC AGA CT Tyr Leu Cys Ala Ser Leu Pro Asp Glu Arg Asn Thr Glu Ala Phe Phe Gly Gln Gly Thr Arg Leu Thr Val
	BV13J1S2	TAC TTC TGT GCC AGC AGC AAT ATT TAC CCC CCG GGA CAG GGG GAT GGC TAC ACC TTC GGT TCG GGG ACC AGG TTA ACC GT Tyr Phe Cys Ala Ser Ser Asn Ile Tyr Pro Pro Gly Gln Gly Asp Gly Tyr Thr Phe Gly Ser Gly Thr Arg Leu Thr
Tumour 2 prog.	BV4J1S2	TAT CTC TGC AGC GTT GTG CTG GGC TAT GGC TAC ACC TTC GGT TCG GGG ACC AGG Tyr Leu Cys Ser Val Val Leu Gly Tyr Gly Tyr Thr Phe Gly Ser Gly Thr Ser
Tumour 2 reg.	BV2J2S5	TTC TAC ATC TGC AGT GGG AAA CTA GAG ACC CAG TAC TTC GGG CCA GGC ACG CGG CTC CTG GTG Phe Tyr Ile Cys Ser Gly Lys Leu Glu Thr Lsn Tyr Phe Gly Pro Gly Thr Arg Leu Leu Val
	BV20J2S5	TAT CTC TGC GCC AGC AGC ACC CCG TGT TCA GGG TGG CAA GAG ACC CAG TAC TTC GGG CCA GGC ACG CGG CTC CTG GTG Tyr Leu Cys Ala Ser Ser Thr Pro Cys Ser Gly Trp Gln Glu Thr Gln Tyr Phe Gly Pro Gly Thr Arg Leu Leu Val
Tumour 3 prog.	BV13J2S7	TAC TTC TGT GCC AAC CGT AGG GCC CTA CTC CAC GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC Tyr Phe Cys Ala Asn Arg Arg Ala Leu Leu His Gln Gln Tyr Phe Gly Pro Gly Thr Arg Leu
	BV14J1S5	TAC TTC TGT GCC AGC AGT TCG ACA GGG AAT CAG CCC CAG CAT TTT GGT GAT GGG ACT CGA GTC TCC TAT CC Tyr Phe Cys Ala Ser Ser Ser Thr Gly Asn Gln Pro Gln His Phe Gly Asp Gly Thr Arg Val Ser Tyr Pro
Tumour 3 reg.	BV6J1S5	TAT CTC CGT GCC AGC AGC TTA ACG CAG CGG GTA TAT CAG CCC CAG CAT TTT GGT GAT GGG ACT CGA CTC TCC ATC CT Tyr Leu Arg Ala Ser Ser Leu Thr Gln Arg Val Tyr Gln Pro Gln His Phe Gly Asp Gly Thr Arg leu Ser Ile
	BV24J2S7	TAC CTG TGT GCC ACC AGC AGA GCC CTA GAA CAG GGG GCG ATT TTC GGG CCG GGC ACC AGG CTC ACG GTC Tyr Leu Cys Ala Thr Ser Arg Ala Leu Glu Gln Gly Ala Ile Phe Gly Pro Gly Thr Arg Leu Thr Val
Tumour 4 prog.	BV14J2S1	TAC TTC TGT GCC AGC AGT TTC GGA CTA GCC CCC TCG TTT GAT GAG CAG TTC TTC GGG CCA GGG ACA AGG GCT CAC Tyr Phe Cys Ala Ser Ser Phe Gly Leu Ala Pro Ser Phe Asp Glu Gln Phe Phe Glu Pro Gly Thr Thr Ala His
Tumour 4 reg.	BV1J2S7	TAT TTC TGT GCC AGC AGG GTA GGG GAC AGG GGA GAT ATA AGG TAC TTC GGG CCG GGC ACC AGG CT Tyr Phe Cys Ala Ser Arg Val Gly Asp Arg Gly Asp Ile Arg Tyr Phe Gly Pro Gly Thr Arg

Table 6. TCRB chain junctional nucleotide sequence and the deduced amino acid sequence. (reg = regressive; prog = progressive) 5′-end V-region and BJ consensus is underlined.

Discussion

Melanoma lesions in many cases show clear signs of regression in some parts whereas other parts of the same tumour show progression (66). Therefore it is possible that progressive samples or progressive parts of the tumours would not give rise to specific HLA-restricted T-cell responses, as two known tumour escape mechanisms are loss of HLA or antigen (153-155). If this is the case, it should not be possible to detect clonal T-cell responses in these parts of the tumours.

Here, we have attempted to answer this question by analysing progressive and regressive parts of four primary tumours. Our data clearly indicate that clonal T-cell expansions take place in both regressive and progressive regions of the tumour and that the T-cell responses directed against the progressive and regressive parts are strikingly different. Recent studies have shown major differences in the BV-expression of TILs when primary tumours were compared with metastatic tumours (108). In the present study, we demonstrate that these differences might actually be present in the primary tumour but become apparent only when different parts of the tumour are analysed separately. In fact, although in some cases the same BV-regions are expressed in both parts of the tumour (i.e. BV13 in tumour 1, BV5 tumour 4) sequence analysis of the CDR3 region showed that they are not derived from the same monoclonal T-cell expansion.

Several explanations can be put forward to account for these marked discrepancies. The differences in BV-region expression may be the result of different antigens expressed on tumour cells present in regressive versus progressive areas. However, it should be noted that different TCR rearrangements are able to react with the same MHC/peptide complex (85; 156; 157). Nevertheless, Sensi and colleagues (158) reported that the majority of cytotoxic T-cell clones recognising MART-1/Melan-A in the context of HLA-$A2^+$ use TCRBV7 or TCRBV14. Recently, this was followed by an analysis of TCRBV in HLA-$A2^+$ primary tumours showing that TCRBV14 was highly expressed in all tumours examined (150). Taken together, these data indicate that although different TCRs may recognise the same HLA/peptide complex, there is strong selection of receptors carrying certain TCRAV/BV chains. In the present study, TIL in three out of four tumours, including two which were HLA-$A2^+$ (tumours 2 and 4), showed expression of TCRBV14. TIL of tumour 4 showed expression of

BV7 in the regressive part and BV14 in the progressive part. In addition, TIL of tumour 2 showed a clonal expansion of TCRBV4, which similarly to TCRBV14 was capable of recognising MART-1/Melan-A in the context of HLA-A2 (158). Thus, it is probable that some of the TCRBV-regions found to be expressed in the present study may recognise antigenic peptides derived from the MART-1/Melan-A protein. This conclusion is supported by the fact that practically all melanomas express this protein (109; 150). It is therefore possible that future studies of TCR usage in HLA typed melanoma lesions may provide further information about the antigens giving rise to the major T-cell responses against melanoma (104).

Although this study is limited to four tumours, the data suggest that T-cell responses may be directed against both regressive and progressive tumour cells. If this is the case, it is likely that HLA or antigen losses are not the major tumour escape mechanism for malignant melanoma. In this regard, it is notable that rather few melanoma cell lines are negative for HLA-expression. We recently analysed 20 melanoma cell lines from 20 HLA-$A2^+$ patients and found only one of them to be HLA-A2 negative (by FACS and RT-PCR) (109). Ferrone et al. (154) suggested the loss of expression of genes involved in transport or processing of the antigen for presentation with HLA class I as a tumour escape mechanism. In this context, we have analysed a large panel of melanoma cell lines for the expression of transcripts coding for TAP1 and 2 as well as LMP2 and 7. In this rather large material, we did not detect any loss of expression for any of these proteins (109). Therefore, this appears to be a highly uncommon mechanism for tumour immune escape in melanoma. Thus, the important question why T cells seemingly capable of rejecting the tumour fail to do so remains elusive.

Detection and characterisation of αβ T-cell clonality by denaturing gradient gel electrophoresis (DGGE)

Abstract

Accumulation of T cells carrying identical TCR is associated with a number of immunological and non-immunological diseases. It is therefore of interest to be able to analyse complex T-cell populations for the presence of clonally expanded subpopulations. Here a simple method is described, combining RT-PCR and denaturing gradient gel electrophoresis (DGGE) for rapid detection and characterisation of T-cell clonality. The detection of clonally expanded T cells by DGGE relies on the fact that clonal transcripts have no junctional diversity and therefore resolve at a fixed position in the gel, determined by their melting properties. For polyclonal populations with a high degree of junctional diversity, the different DNA molecules will resolve at different positions in the gel and together will be revealed as a smear. For each of the TCR families BV1-24, cloned transcripts were amplified and shown to resolve as distinct bands in the denaturing gradient gel, whereas the analysis of polyclonal T-cell populations resulted in a smear.

The present method may prove useful to test for clonotypic T cells in a variety of pathological and physiological conditions, and for monitoring T-cell responses in diagnostic and therapeutic settings.

Introduction

A number of different methods exist for the detection of T-cell clonality. The most widely used methods are based on RT-PCR followed by single strand conformation polymorphism (SSCP)(159), PCR heteroduplex analysis (160) or CDR3 size determination (161). However, all these methods require steps of either blotting and hybridisation or reamplification using end-labeled probes followed by computerised data analysis. In the present study, we have developed a method based on RT-PCR and denaturing gradient gel electrophoresis (DGGE) to rapidly screen for the presence of clonal T cells. The method detects the presence of T-cell clonotypes, covering the BV regions 1-24, and facilitates the possibility to produce full and detailed "TCR clonotype maps".

DGGE is a powerful method for the detection of small deletions, small insertions and point mutations, having detection efficiency close to 100% (162). The method relies on the fact that the melting properties of a DNA molecule are highly dependent on the nucleotide composition. During electrophoresis in a denaturing gradient gel, the DNA molecule will partially melt at a sequence-dependent concentration of denaturants, and the resulting partial separation of the DNA duplex will retard the molecule and thereby reveal a distinct band in the gel. This implies that even single base pair changes in a DNA sequence can be revealed in a denaturing gradient gel by a shift in the position at which the molecule is retarded (162). In a polyclonal T-cell population, all TCR DNA sequences will, in theory, differ from each other in their melting properties and will therefore be revealed as a smear in the denaturing gradient gel. In contrast, any population of clonally expanded T cells will be revealed as a distinct band which can be recovered for further analysis.

Materials and methods

In vitro culture of peripheral blood lymphocytes

Samples of heparinised blood were obtained from healthy volunteers. Lymphocytes were separated by centrifugation on Lymphoprep (Nycomed, Oslo, Norway) and cultured in RPMI-1640 containing 10% FCS for four days in the presence of 20 μg/ml phytohemagglutin-P (PHA) (Sigma, ST. Lous. MO, USA) for polyclonal stimulation, or 25 μg/ml purified protein derivative (PPD) (Statens Seruminstitut, Copenhagen, Denmark) for specific stimulation. Cells were stored as a pellet at -80°C, or RNA was isolated immediately.

Primer sequences and melting maps

The sequences of the primers used for the amplification of TCRBV regions 1-24 are given in Table 7. Sequences of TCRBV regions were obtained from the GenBank Database using accession numbers given by Arden et al. (23). Using the PC/GENE program "CLUSTAL" (Intelligenetics, Palo Alto, CA, USA) the sequences in each family were aligned, and primer sequences were found in regions of optimal consensus. The selection of optimal primers were aided by the computer program Oligo, Version 3.4 (Medprobe, Oslo, Norway) (130), aiming at a ΔG value of approximately -40.0 and a T_m between 50°C and 60°C. Melting maps for amplified PCR products were calculated using the computer algorithm MELT87 (163).

RT-PCR

RNA was extracted using the Purescript Isolation Kit (Gentra Systems Inc. NC, USA). cDNA synthesis was carried out using 1-3 μg of total RNA as described (60). TCR cDNA was quantitated using primers specific for the constant part of the β chain (BC5'and BC3') as described (136). Using the same amount of TCR template in each amplification, cDNA was amplified using primers specific for TCRBV regions 1-24 together with the "GC-clamped" constant region primer BCseq2. Amplifications were carried out in a total volume of 25 μl as described (136).

DGGE

Gels for DGGE analysis contained 6% polyacrylamide and a gradient of urea and formamide from 20% to 80%, and were run at 160 V for 4.5 h in 1x TAE buffer kept at a constant temperature of 58°C. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV transillumination.

In order to demonstrate that all products could be resolved in the gel, PCR products for TCRBV1-24 were cloned using the TA-cloning kit (Invitrogen, San Diego, CA, USA) following the manufacturer's instructions. Positive bacterial clones were PCR amplified for 30 cycles with the specific BV-primer together with the "GC-clamped" BC primer, and 10 µl aliquots were loaded onto the gel.

Quantitation of clonotypic transcripts

To investigate the detection sensitivity of the method, PCR was carried out using a ^{33}P labeled BCseq2-GC primer amplifying PBL cDNA intermixed in different ratios with a clonal TCR transcript (BV17). Samples were loaded twice onto a denaturing gradient gel; the second loading was performed 30 min before the running time expired, enabling quantitation of the amount of clonal transcript (first loading) and the total amount of TCR transcript (second loading). Quantitation was carried out using the ImageQuant software. Relative abundance of the clonotypic transcript was calculated as clonotypic signal/total signal x 100.

Results

Primer sequences

Primers were selected aiming at a match to all members of the BV family in question and 5 mismatches to all other families. Furthermore, the amplified products were chosen to be within a limited size range (Table 7). Some BV families (BV5, BV6, BV12, and BV13) were amplified using two different BV primers in order to match all members of these BV families. Because the amplification of BV13 was inhibited by the presence of both BV13-primers in the same tube, amplification of the BV13 family was carried out in two different reaction tubes.

Melting maps

Using the computer algorithm MELT87 melting maps for each amplified TCRBV region were calculated for the theoretical ability to resolve using standardised conditions. For most of the 24 BV-regions a suitable melting profile could be obtained by the attachment of a 50 bp "GC-clamp" to the 5′-end of the constant region primer BCseq2 (Fig 7). A short "GC" clamp (8-16 bp) was added to the 5′-end of some BV-primers (BV1 and BV21) to modulate the melting profile (Table 7).

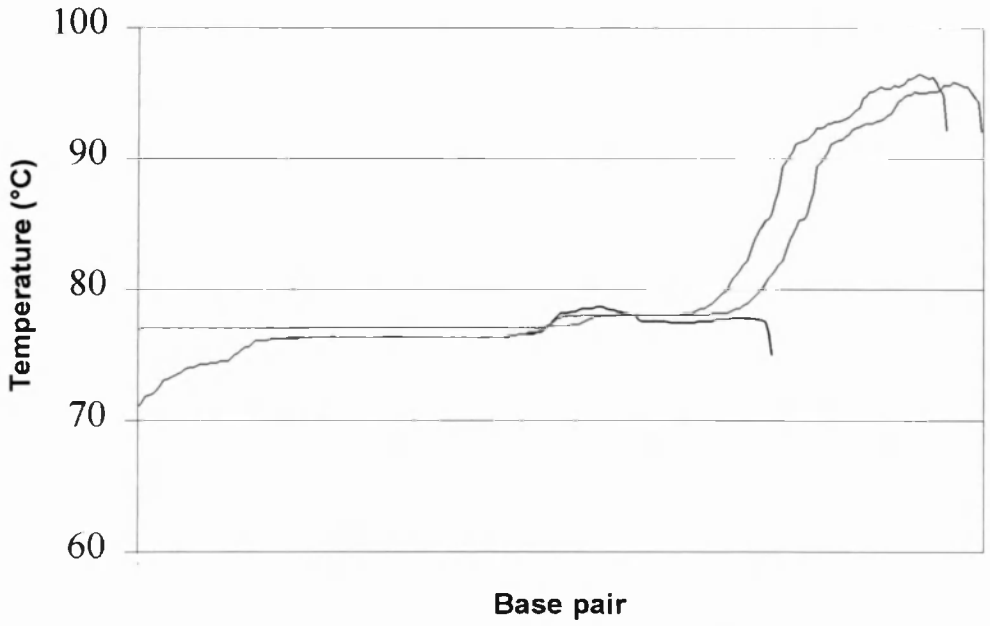

Figure 7. Melting profile of a BV21 PCR product without clamp (black), with 50 bp 3′-clamp (green) and with both 5′- and 3′-clamp (red) (Table 7).

Table 7. TCRB Oligonucleotide primers for DGGE analysis.

BV	Sequence	Pos.	Size	ΔG	T_m
BV1	5′-(GC^6)-ACTCTGAACTAAACCTGA-3′	-55	199	-41.3	51.2
BV2	5′-TCAACCATGCAAGCCTGACC-3′	-74	212	-39.3	55.7
BV3	5′-CGCTTCTCCCTGATTCTGGAGTCC-3′	-54	192	-47.1	61.2
BV4	5′-TTCCCATCAGCCGCCCAAACCTA-3′	-81	219	-49.9	65.0
BV5	5′-CTGAGATGAATGTGAGCACCTTG-3′	-51	189	-39.8	53.9
	5′-CTGAGCTGAATGTGAACGCCTTG-3′	-51	189	-43.4	58.6
BV6	5′-AGATCCAGCGCACAGAGCG-3′	-41	179	-39.3	56.2
	5′-AGATCCAGCGCACASAGCA-3′	-41	179	-37.6	54.9
BV7	5′-GCCAAGTCGCTTCTCACCTG-3′	-90	228	-39.3	54.8
BV8	5'-TGAAGATCCAGCCCTCAGAACCC-3'	-43	181	-45.3	60.2
BV9	5′-CTTCACATCAATTCCCTGGAGA-3′	-45	183	-39.7	53.1
BV10	5′-CCACGGAGTCAGGGGACACA-3′	-32	170	-39.9	57.8
BV11	5′ TGCCAGGCCCTCACATACCTCTCA-3'	-31	169	-47.4	63.5
BV12	5′-GAGAATTTCCTCCTCACTCTGG-3′	-47	185	-39.2	51.2
	5′-GACCTCCCCCTCACTCTGG-3′	-44	182	-37.8	53.0
BV13	5′-CTCAGGCTGCTGTCGGCTG-3′	-45	183	-39.2	56.8
	5′-CTCAGGCTGGAGTTGGCTG-3′	-45	183	-37.5	53.1
BV14	5′-AGGGTACAAAGTCTCTCGAAAAG-3′	-84	222	-40.4	50.7
BV15	5′-CAGGCACAGGCTAAATTCTCC-3′	-65	203	-40.2	53.1
BV16	5′-(GC^{11})-GGCGAACTGGAGGATTCTGGAGT-3′	-30	181	-42.0	55.4
BV17	5′-GAAGGGTACAGCGTCTCTCGG-3′	-87	225	-41.0	55.3
BV18	5′-TTTCTGCTGAATTTCCCAAAGAGG-3′	-81	219	-45.7	57.7
BV19	5′-TCTCAATGCCCCAAGAACGCAC-3′	-74	212	-44.5	60.8
BV20	5′-AGGTGCCCCAGAATCTCTCAG-3′	-95	233	-40.3	54.7
BV21	5′-(GC^8)-GCTCAAAGGAGTAGACTCCACTCTC-3′	-65	211	-42.5	53.9
BV22	5′-AGATCCGGTCCACAAAGCTG-3′	-35	173	-39.2	54.0
BV23	5′-ATTCTGAACTGAACATGAGCTCCT-3′	-53	191	-41.1	53.0
BV24	5′-ATCCAGGAGGCCGAACACTTC-3′	-77	215	-42.0	56.8
BCseq2	5′-ACAGCGACCTCGGGTGGGAA-3′	38		-43.4	61.2
BC5′	5′-TGAGCTGGTGGGTGAATGGG-3′	187	343	-40.5	57.9
BC3′	5′-GGTGGCCTTCCCTAGCAGG-3′	530		-40.7	55.9
BCseq2-GC					
5'-GCCGCCCGCCGCTCGCCCGCCGCGCCCCTGCCCGCCGCCCCCGCCCGACAGCGACCTCGGGTGGGAA-3					

Position +1 is defined as the first nucleotide after the sequence coding for the conserved amino acid sequence CASS in the proximal end of the variable domain. The approximate sizes of the PCR products are calculated using an estimated mean length of the DJ region of 50 bp.

DGGE analysis of cloned TCRBV1-24

Cloned TCR transcripts were PCR amplified and subjected to DGGE analysis to investigate whether it was possible to resolve all 25 products in the same gel using standardised conditions. For each TCRBV family several individual clones were amplified, and a minimum of 4 different clones were evaluated by DGGE. In all cases it was possible to resolve the product as a distinct band in the gel (Fig. 8).

To investigate the ability of the method to distinguish between different clones carrying the same TCRBV region, 16 different TCRBV13 clones were amplified and run in the same gel (Fig. 9). The

results clearly indicate that the method is capable of distinguishing between clones that differ only in the junctional region of the receptor.

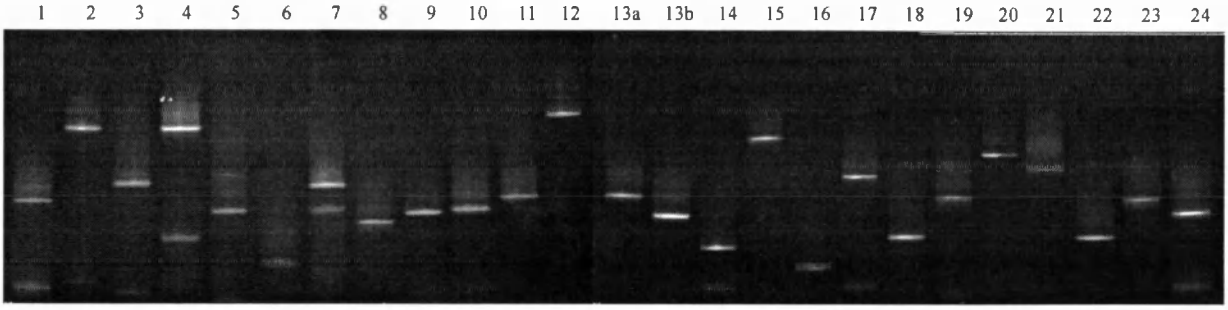

Figure 8. Denaturing gradient gel analysis of TCR transcripts covering BV families 1-24. PBL cDNA was amplified with primers specific for BV families 1-24, cloned into PCR^{TMII}, reamplified and loaded onto a 20% - 80% denaturing gradient gel and run for 4.5 hours at 160 Volts. DNA was stained with ethidium bromide and photographed under UV light.

Analysis of PHA and PPD stimulated T cells

To verify that polyclonal T-cell populations will reveal a smear, whereas the presence of clonotypic T cells will reveal a distinct band in the gel, T cells were stimulated with PHA and PPD respectively, and analysed using DGGE. Prior to DGGE analysis it was tested by agarose gel electrophoresis that all 25 reactions had successfully amplified products of the correct size without spurious amplification products. The results showed that the RT-PCR/DGGE analysis of polyclonal T cells results in a gel smear whereas the analysis of PPD stimulated T cells demonstrated the presence of several distinct bands in the gel. Examples of the DGGE analysis of PHA and PPD stimulated T-cell populations are seen in Fig 10.

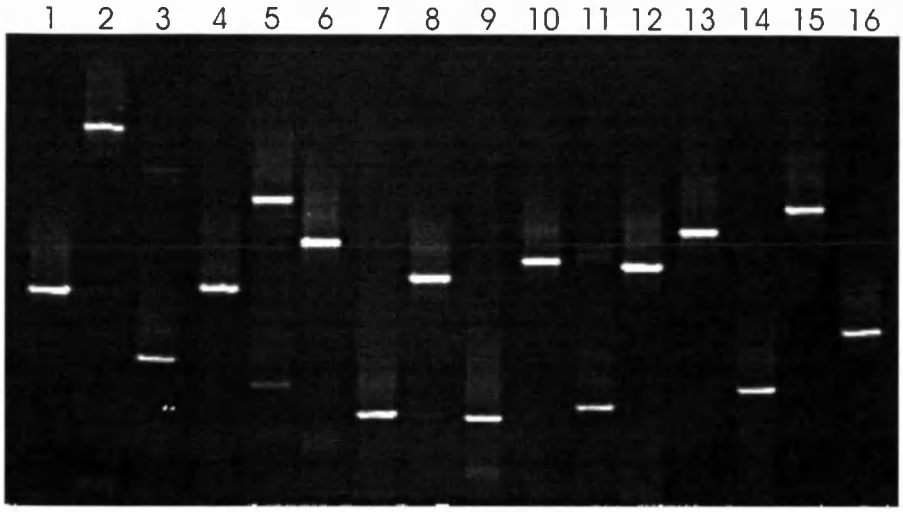

Figure 9. DGGE analysis of sixteen cloned TCRBV13 transcripts. cDNA was prepared from PHA activated lymphocytes and the BV13 transcripts were amplified. Subsequently these BV13 PCR products were cloned into PCR^{TMII}, reamplified and loaded onto a 20% - 80% denaturing gradient gel and run for 4.5 hours at 160 Volts. DNA was stained with ethidium bromide and photographed under UV light.

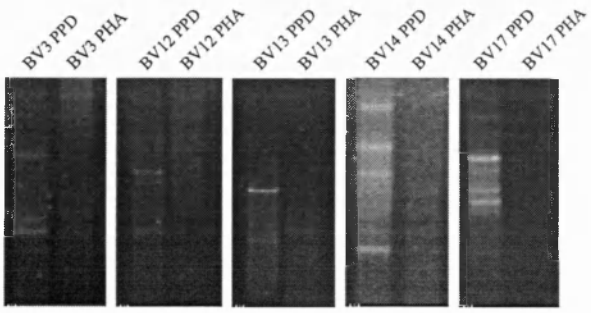

Figure 10. DGGE analysis of T cells stimulated with PPD or PHA. The individual amplifications were carried out using equal amounts of TCR cDNA.

Quantitation of clonotypic transcripts in a heterogeneous background

To investigate the detection level of DGGE, experiments were carried out to quantitate the fraction of a clonotypic transcript in a mixed T-cell population. This was achieved by mixing at different ratios cDNA derived from a T-cell clone with cDNA from PBL. Dual loading at different timepoints enabled the quantitation of the clonotypic BV17 transcript relative to the total amount of TCRBV17 (Fig. 11). This analysis showed that it is possible to detect clonal transcripts constituting below 2.5% of the total amount of PCR product.

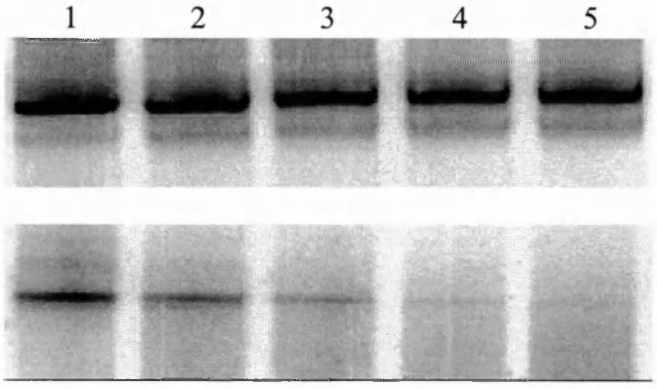

Figure 11. Sensitivity of the DGGE method. Clonal TCR cDNA was mixed with cDNA from PHA stimulated T cells to enable the quantitation of clonal and polyclonal components in the sample. Upper panel shows the sample after 30 min DGGE (prior to sequence dependent separation); lower panel shows the sample after 4.5 hour DGGE (after sequence dependent separation) resolving only the clonal transcript as a distinct band in the gel. The clonal transcript in the first four lanes was visible under UV light, after staining with EtBr.

Discussion

We have here described a novel approach for detection of αβ T-cell clonality. The method combines RT-PCR to selectively amplify 24 TCRBV families, and DGGE to resolve clonotypic T-cell sequences. Calculation of melting profiles of randomly selected TCR β sequences formed the basis for selection of BV primers that together with a "GC-clamped" constant region primer would amplify all relevant sequences and render them suitable for DGGE analysis. The versatility of the assay was demonstrated by the absence of bands in the gel when polyclonal populations were analysed. In contrast, the analysis of specifically activated T cells, revealed the presence of numerous bands in each lane; the full analysis providing "TCR clonotype maps" of the analysed T-cell population. Distinct bands in the gel were detected when cloned transcripts from each of the 24 different TCRBV families were analysed. Several cloned BV products from each family were analysed using DGGE, and all of these were resolved as distinct bands in the gel. Furthermore, the analysis of 16 different cloned transcripts derived from the TCRBV13 family indicated that the present method has the resolving power to distinguish between most clonal transcripts derived from the same TCRBV family. Obviously, situations may occur in which two different transcripts retard too close to each other to be recovered from the gel or even be distinguished as separate bands using "broad range" DGGE parameters (164). Our analysis of cloned TCR transcripts nevertheless indicates that it is a rare situation to have two different sequences that cannot be clearly distinguished as separate bands. This also makes the present method ideal for analysis of *in vitro* established T-cell clones carrying the same BV region. When the amplified products are run in the same gel, the specific individual band pattern will reveal whether or not the clones are sister clones.

Most analyses of T-cell clonality focus on the examination of pathological tissues for the presence of clonotypic T cells. An important aspect is therefore related to the sensitivity of the method. Using DGGE, clonotypic transcripts that constitute only a minority of the T cells carrying the specific BV-region will be revealed in the gel. Clonotypic transcripts constituting as low as 2.5% of the BV region in question can easily be visualised in the gel by ethidium bromide staining. Assuming that all regions are expressed at equal levels, this means that a T-cell clone can be detected in a mixed population at a fraction of 0.1 %.

At present, all methods for the analysis of clonality in TCRBV regions involve different steps of either blotting, re-amplification, radioactive labeling or exposure to autoradiographs (159-161; 165). The DGGE based method described here requires none of these steps and visualisation of the amplified DNA is accomplished by means of simple staining with EtBr. The method is therefore simple and fast and includes steps that can easily be set up for routine analysis, even in more modestly equipped laboratories. The described method may provide a powerful tool to monitor T-cell responses in connection with vaccination protocols, immunotherapeutic transfusion of T cells, and other situations where a T-cell clone is present as a minority in an otherwise polyclonal T-cell population.

Local *versus* systemic immune reactions against disseminated melanoma: predominance of localised clonotypic T-cell expansions

Abstract

It is well established that melanoma cells express antigens that are recognised by autologous T-cells. In terms of the *in vivo* T-cell response, current insight is however limited, and little is known with regards to the detailed characteristics of the T-cell response. In the present study we have used TCR clonotype mapping for detailed characterisation of the *in situ* T-cell response against human melanoma.

Analysis of six subcutaneous metastases from two patients revealed the presence of multiple clonotypic T-cells in all lesions, the number of T-cell clonotypes ranging from thirty to more than forty in each lesion. The same lesions were analysed for the relative expression of TCRBV regions 1-24 demonstrating that clonotypic T cells were present in regions expressed at both high and low levels. Comparison of the T-cell clonotypes present in the three lesions from each patient demonstrated that most clonotypes were exclusively detected in a single lesion.

The present data indicate that *in vivo* anti-melanoma T-cell responses are much more heterogeneous than previously appreciated and accommodate a predominance of strictly localised T-cell clonotypes.

Introduction

Melanoma cells are considered to be immunogenic and T cells often infiltrate melanoma lesions. Although the direct evidence of the therapeutic significance of these T cells is still lacking, a number of clinical observations, e.g. the higher risk of melanoma for immune-suppressed individuals and the induced regression of metastatic lesions subsequent to immunotherapy (128; 129), strongly indicate a functional relevance of the T cells. It has been shown that the immune responses to melanoma include CTL capable of lysing autologous melanoma cells *in vitro*, and recently a number of melanoma associated antigens have been characterised and the peptide epitopes defined (65). However, it is not known whether these antigens are capable of inducing T-cell responses *in vivo*. Analyses of the *in vivo* T-cell response have suggested the involvement of clonotypic T cells in the immunological response to malignant melanoma. Still, most studies have been limited to the analysis of T-cell clonality in sub-populations of TIL expressing specific TCRBV regions at a significant level (102; 103). Consequently, current knowledge is limited in terms of the heterogeneity of the T-cell infiltrate and the numbers of T-cell clones engaged with *in vivo* responses against melanoma.

Complete and detailed analyses have been hampered by the lack of methods capable of detecting clonotypic T cells with high sensitivity in a heterogeneous background. In the present study, we have used a recently developed, highly sensitive method based on RT-PCR and DGGE to analyse for the presence of clonotypic T cells in six subcutaneous melanoma lesions from two patients. We demonstrate that the T-cell infiltrate in melanoma is exceedingly heterogeneous and accommodate a much larger number of T-cell clonotypes than previously appreciated. Using a semi-quantitative RT-PCR methodology we further examined the TCRBV repertoire and established that T-cell clonotypes may be detected in BV regions expressed at high as well as low levels. Furthermore, we demonstrate that any specific T-cell clonotype is in most cases exclusively present in a single lesion only, indicating that the T-cell infiltrate in melanoma recruits a predominance of localised clonotypic T cells.

Materials and Methods

Tumour Samples

Fresh subcutaneous melanoma lesions were received as biopsy material immediately after surgery performed at the Department of Plastic Surgery, University Hospital, Copenhagen (patient #1) and Department of Dermatology, School of Medicine, D-97080 Würzburg, Germany (patient #2). PBL were used for tissue typing. Tumour lesions and blood samples were taken from the respective patients at the same time point.

Immunohistology

Frozen sections were fixed in cold acetone for 10 minutes followed by removal of endogenous peroxidase with 0.03% H_2O_2, and blocking of collagenous elements with 10% species specific serum in 1% BSA/PBS. The antibodies were then overlayed onto serial sections, at predetermined dilutions (usually 20 μg/ml) and the slides were incubated in a humid chamber for 30 minutes. With PBS washes between every step, a biotinylated link antibody was applied for 10 minutes followed by a streptavidin-linked enzyme, i.e. either peroxidase or alkaline phosphatase, for 10 minutes. After another wash, the substrate was added and the slides were incubated in the dark for 20 minutes. After a wash in PBS, the slides were counter stained, mounted and viewed using an Olympus BH2 microscope with photographic capabilities. Anti-Vβ13 and Vβ14 antibodies are commercially available and used according to manufacturer's guidelines (Immunotech, Westbrook, ME, USA).

RT-PCR for quantitative TCRBV analysis and TCR clonotype mapping

Tumour biopsies and blood samples were processed immediately after surgery. RNA was extracted using the Purescript Isolation Kit (Gentra Systems Inc. NC). cDNA synthesis and quantitation of TCR cDNA in the each sample were carried as described (136). Using equal amounts of TCR template in all reactions cDNA was amplified using separate primer panels for quantitative TCRBV region analysis (136) and for TCR clonotype mapping (166). Amplifications were carried out in a total volume of 25 μl containing 1xPCR buffer (50mM KCl, 20 mM Tris pH 8.4, 2.0 mM $MgCl_2$, 0.2 mM

cresol red, 12% sucrose, 0.005% (w/v) BSA (Boehringer-Mannheim, Mannheim, Germany)), 2.5 pmol of each primer, 40 mM dNTPs (Pharmacia LKB, Uppsala, Sweden) and 1.25 units of AmpliTaq polymerase (Perkin Elmer Cetus Corporation, Emeryville, CA.). Parameters used for the amplification were 94°C for 60 sec, 60°C for 60 sec. and 72°C for 60 sec. for 32 (quantitative PCR) or 40 cycles (DGGE). Taq polymerase and dNTPs were added to the reaction tube at an 80°C step between the denaturation and annealing steps of the first cycle (Hot start).

For quantitative analysis, the constant region primer was labeled with ^{33}P. Ten-microliter aliquots of PCR products were electrophoresed in a 2% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME) which was subsequently dried under vacuum and exposed to a Molecular Dynamics Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA). Quantitation was accomplished using the Imagequant software (137).

For DGGE analysis, 10µl aliquots were loaded onto a denaturing gradient gel containing 6% polyacrylamide and a gradient of urea and formamide from 20% to 80%. Gels were run at 160 V for 4.5 h in 1x TAE buffer kept at a constant temperature of 58°C. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV transillumination.

To analyse for the expression of Tyrosinase, MART-1/Melan-A and gp100, RT-PCR was carried out with primers specific for these transcripts, as previously described (109).

Sequencing

To verify sequence identity to DNA bands that resolved at similar positions in the denaturing gradient gel, these transcripts were subjected to sequence analysis using the Thermo Sequenase cycle sequencing kit (Amersham, Life Science, Cleveland, OH). In brief, bands were excised from the denaturing gradient gel, and DNA was eluted in H_2O and reamplified. An aliquot (0.2 µl) of the PCR product was used as template in a 40-cycle sequencing reaction with ^{33}P labeled Bcseq2 as sequencing primer. Gels were dried under vacuum and exposed to a Phosphor Screen.

Results

RT-PCR for MAAs

To investigate whether the lesions exhibited any differences in the expression of MAA, the six biopsies were analysed for the expression of Tyrosinase, MART-1 and gp100 by RT-PCR. All lesions were positive for the expression of these transcripts (data not shown).

TCRBV expression

PBL and three subcutaneous melanoma lesions from each patient were analysed for the expression of TCRBV regions 1-24 by semi-quantitative RT-PCR. The results from these analyses are given in Table 8. In all lesions, only a limited number of BV regions were expressed at levels significantly above what was seen in the PBL from the same patient. However, major differences were detected in the level of BV region expression between the different lesions from each patient (e.g. patient #1, BV6; 2.8, 10.1 and 6.0% respectively in lesion 1, 2, and 3; patient #2, BV3, 6.6, 10.6, and 3.4% respectively in lesion 1, 2, and 3). Although the data for the PBL did not directly indicate the presence of clonally expanded T cells in the tumours, the comparison of the different lesions could indicate distinct T-cell populations in each lesion.

The patients PBL were used for histological tissue typing (Patient #1 : HLA-A1, A2, B8, B44, Cw3, Cw5, DR3, DR9, Patient #2 : Not done).

TCR clonotype mapping

PBL and tumour lesions were analysed for the presence of T-cell clonotypes by the DGGE based TCR clonotype mapping. Analysis of PBL did not reveal any distinct bands in the denaturing gradient gel (Fig. 13, and data not shown). Analysis of tumour samples, however, showed the presence of multiple clonotypic TCR transcripts covering the majority of the TCRBV regions 1-24. In all lesions more than thirty clonotypes were detected and in lesion 3 from patient #2 an exceedingly high number of clonotypes was detected (Fig. 12, and Table 8). Comparing the number of clonotypes and the relative

Table 8.

	Patient #1							Patient #2						
	Metastasis 1		Metastasis 2		Metastssis 3		PBL	Metastasis 1		Metastasis 2		Metastasis 3		PBL
TCRBV	Exp. %	Clones	Exp. %	Clones	Exp. %	Clones	Exp. %	Exp. %	Clones	Exp. %	Clones	Exp. %	Clones	Exp. %
1	3.7	3	5.7	3	4.6	>4	4.1 (0.2)	3.5 (1.0)	0	8.4 (1.3)	4	8.1 (0.0)	3	9.2 (1.1)
2	4.1	1	7.9	>4	3.0	2	3.3 (0.5)	2.7 (0.0)	3	5.4 (1.6)	1	5.0 (0.3)	3	5.9 (1.5)
3	6.4	2	7.4	2	5.2	>4	7.0 (0.0)	6.6 (1.4)	>4	10.6 (0.4)	4	3.4 (0.3)	3	3.1 (1.6)
4	5.4	>4	9.8	4	2.3	3	3.3 (0.7)	6.4 (0.2)	2	4.0 (0.0)	3	7.9 (1.1)	2	5.7 (0.4)
5	4.0	2	9.3	2	4.5	4	3.8 (0.1)	9.0 (1.6)	>4	7.6 (2.8)	0	3.9 (0.0)	0	4.6 (2.2)
6	2.8	0	10.1	0	6.0	2	4.2 (1.7)	4.7 (0.1)	0	4.0 (1.0)	0	6.9 (0.1)	0	3.9 (0.2)
7	<1	0	<1	0	<1	2	1.5 (0.5)	<1 (0.0)	0	<1 (0.1)	0	<1 (0.1)	0	4.6 (0.6)
8	6.7	>4	9.5	3	2.9	>4	6.2 (0.7)	8.1 (0.5)	0	9.1 (0.7)	4	9.0 (2.1)	4	3.3 (0.4)
9	3.5	0	2.9	0	3.1	4	4.0 (0.3)	2.5 (0.0)	0	6.3 (2.5)	0	1.8 (0.1)	2	4.4 (0.5)
10	5.6	2	<1	0	3.5	1	4.0 (0.6)	7.3 (0.2)	4	<1 (0.1)	0	1.4 (0.1)	0	3.7 (0.4)
11	<1	2	<1	0	3.0	3	2.9 (0.6)	6.7 (1.4)	1	<1 (0.1)	0	<1 (0.1)	1	2.6 (0.8)
12	3.8	1	<1	1	3.5	4	4.8 (0.8)	4.5 (1.6)	>4	<1 (0.2)	1	7.2 (1.7)	3	3.9 (1.6)
13	4.2	>4	3.8	3	2.7	>4	4.3 (1.9)	4.2 (1.2)	>4	7.3 (0.2)	<4	5.1 (0.7)	3	7.5 (0.7)
14	6.3	>4	1.1	2	3.8	2	3.7 (0.3)	3.9 (1.1)	4	2.5 (0.0)	2	3.6 (1.0)	2	3.4 (0.8)
15	1.5	3	<1	0	1.5	>4	2.5 (0.2)	<1 (0.1)	1	1.5 (0.1)	1	<1 (0.6)	1	1.5 (0.8)
16	3.8	2	4.3	1	4.6	>4	3.2 (1.5)	2.6 (0.0)	0	1.0 (0.3)	0	1.6 (0.4)	1	2.7 (0.5)
17	2.9	>4	<1	1	2.8	0	4.0 (0.2)	2.1 (0.3)	3	2.9 (0.1)	1	1.1 (0.2)	1	5.4 (0.8)
18	2.8	0	2.3	0	2.9	0	4.5 (0.5)	1.9 (0.2)	0	5.3 (1.3)	0	1.9 (1.2)	2	3.4 (0.8)
19	4.1	1	<1	0	4.4	3	4.3 (0.2)	3.4 (0.3)	0	6.5 (1.2)	0	<1 (0.1)	3	2.2 (0.3)
20	7.1	3	8.1	4	8.6	4	6.5 (0.4)	<1 (0.0)	0	8.6 (0.7)	2	7.2 (1.0)	2	2.0 (0.1)
21	4.6	2	4.1	>4	6.3	1	6.4 (1.3)	5.4 (2.1)	4	5.3 (0.3)	2	6.4 (0.6)	1	7.3 (1.0)
22	8.2	>4	7.6	2	8.6	1	5.0 (2.4)	6.3 (0.1)	3	2.1 (0.2)	2	6.9 (0.1)	3	4.3 (1.8)
23	2.7	>4	<1	0	4.2	3	1.9 (0.3)	2.8 (0.0)	0	<1 (0.0)	0	2.7 (0.1)	0	5.1 (0.2)
24	5.1	3	3.3	1	7.2	>4	4.4 (1.2)	3.7 (1.1)	3	<1 (0.0)	4	7.3 (0.3)	1	0.9 (0.0)

TCRBV usage by quantitative RT-PCR and number of T cell clonotypes by TCR clonotype mapping, in subcutaneous melanoma lesions from patients #1 and #2. Each BV-region is expressed as (mean (SD)) percentage (Exp. %) of the total TCRBV signal. Values lower than 1% is indicated as < 1.
When more than 4 clonotypic transcripts were detected this is disignated as > 4. No clonal transcripts were detected in the PBLs.

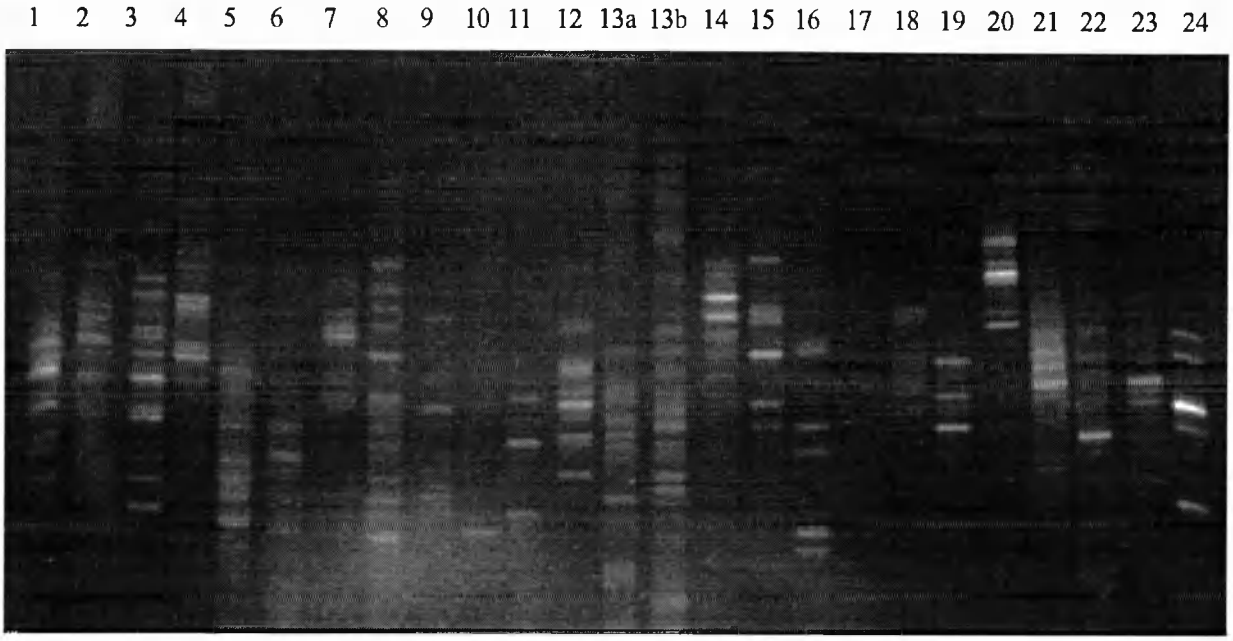

Figure 12. TCR clonotype maps of melanoma biopsy. cDNA from tumour tissues and peripheral blood lymphocytes were amplified with primers specific for BV families 1-24 and aliquots loaded onto a 20% - 80% denaturing gradient gel and run for 4.5 hours at 160 Volts at a constant temperature of 58°C.

expression of the different BV regions clearly demonstrated that clonotypic transcripts were detected in regions expressed at high as well as low levels.

To investigate whether specific T-cell clonotypes could be detected in more than a single lesion, the DGGE analysis was repeated and samples loaded in adjacent lanes of the denaturing gradient gel. As depicted in Fig. 13, most of the clonotypes were exclusively present in a single lesion. More than fifty clonal TCR transcripts were compared of which six appeared to be accumulated in two different lesions. None of the clonotypes were present in all three lesions from the same patient.

Sequencing

A number of transcripts were detected that resolved at positions in the gel indicating sequence identity (Fig. 13). These DNA bands were therefore subjected to sequence analysis, including Patient #1: BV4 – lesion 1 (lower band) and lesion 3 (lower band), different sequences; BV16 – lesion 1 (upper band) and lesion 3 (second upper band), different sequences; BV22 – lesion 1 (lower band) and lesion 2 (lower band), identical sequence; BV22 - lesion 1 (second lower band) and lesion 3 (lower band),

identical sequences; BV24- lesion 2 and 3, (second and third lower band respectively), identical sequences. Patient #2: BV14 – lesion 1 (second lower band), lesion 3 (lower band), identical

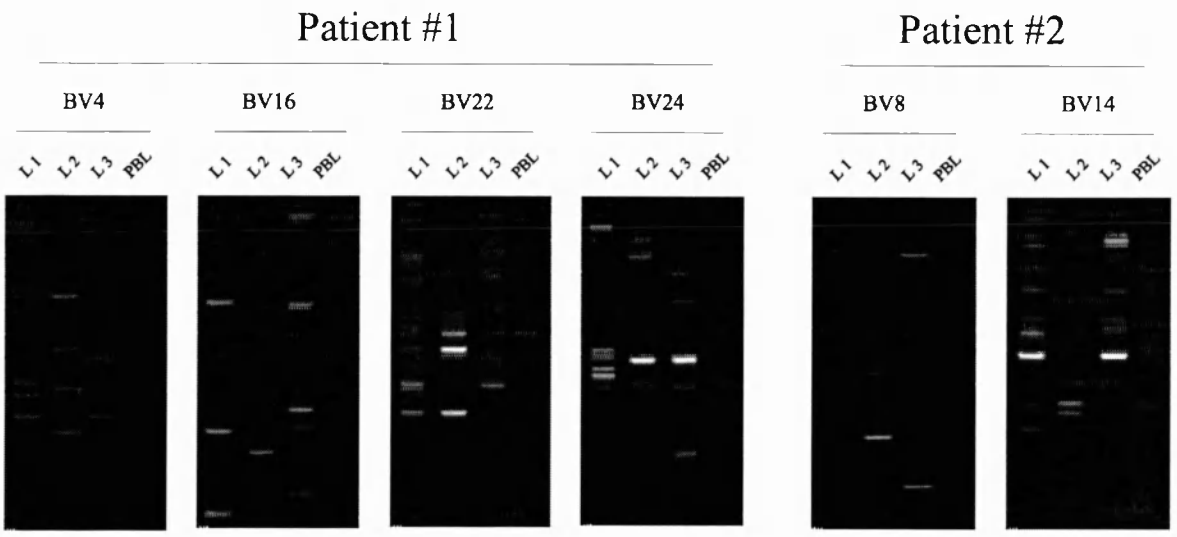

Figure 13. Comparative DGGE analysis of specific T-cell clonotypes in lesions 1-3 (L1-L3) from the two patients

sequences). Out of six potentially identical sequences, only four of these were indeed identical. It should be noted that the two different BV4 sequences derived from patient #1, lesions #1 and #3, shared the same joining sequence, were of identical length, and only differed in the junction between the variable and the joining region.

Immunohistology

The immunological staining with monoclonal antibodies specific for BV13 or BV14 in lesion #3 from patient #1 demonstrated that positive cells were cells not present through out the tumor, but present as clusters of cells. Only a limited number of singly positive stained cells were present indicating that the T cells are activated and expand but do not migrate to other parts of the tumor.

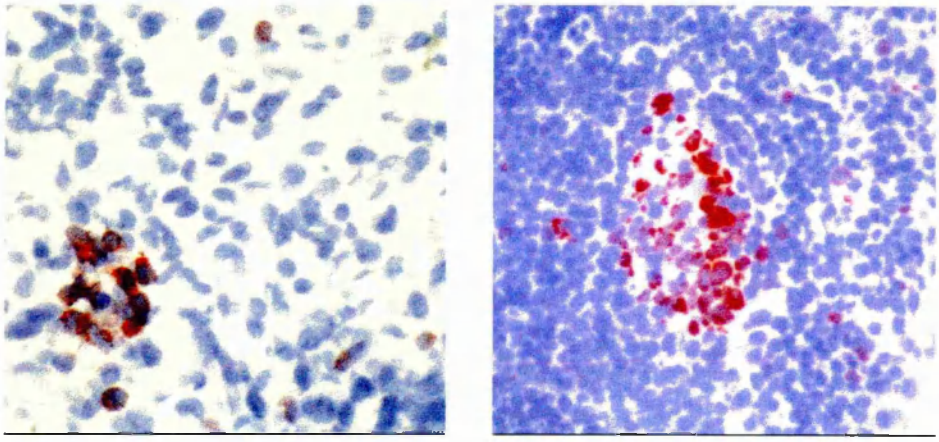

Figure 14. Immunohistology of lesion #3 from patient #1, using anti-Vβ13 (left) or anti-Vβ14 specific antibody.

Discussion

During the past decade important insights has been unveiled into the role of T lymphocytes in the host's immune response to cancer in general and to melanoma in particular (64). A number of melanoma associated antigens have been characterised which are specifically recognised by autologous T cells in the context of HLA molecules (65) and precursor T cells specific for melanoma associated antigens, have been isolated from PBL of melanoma patients (72). Several clinical observations, e.g., the immunologically mediated partial regression of primary melanoma (167) and the higher risk of immune suppressed individuals for malignant melanoma (168), indicate the relevance of the host immune responses for the control of melanoma progression. The notion that a functional and specific T-cell response may be operating in melanoma patients was reinforced by the demonstration of clonotypic T cells in both primary and metastatic melanoma lesions (108; 150). Although our knowledge of the T-cell response against melanoma has increased considerably, only few attempts have been made to characterise in detail the complexity of the *in vivo* T-cell response and very little is known about the number of clones involved in the T-cell response and the TCRBV regions expressed by these clones. It therefore remains elusive whether specific clones or BV families participate in a systemic T-cell response against tumour burden at different anatomical sites in the same patient.

In the present study we have attempted to answer some of these questions using TCR clonotype mapping. The analysis of the melanoma lesions revealed the presence of multiple clonotypic TCR transcripts (Fig. 12), the numbers of clonotypes ranging from approximately thirty up to more than forty (Table 8).

Previous studies have been limited to analyse for the presence of clonotypic T-cells in highly expressed BV regions (102; 105; 150). In contrast, here we analysed the BV regions 1-24, not discriminating by the level of expression. This approach revealed that clonal T cells do exist in most BV regions irrespective of the level of expression (Table 8). Puisieux et al. (169) analysed melanoma lesions for the presence of T-cell clonality by determining the VDJ junction size patterns of TIL. This approach did not reveal the same degree of clonotypic complexity as in the present study, suggesting that VDJ junction size patterns may not always reflect the true number of individual clonal

expansions. When several clones carrying the same BV region are present in a T-cell population, the accurate detection of clonality by determining the VDJ junction sizes may be hampered. TCR clonotype mapping separates clonotypic transcripts according to their melting properties, implying that transcripts, which differ in primary sequence, will be resolved at different positions in the gel independently of the length of the transcript.

The present data suggests that the *in situ* T-cell response against malignant melanoma is highly complex and involves a much higher number of T-cell clones than previously appreciated. By repertoire analysis (170) or more recently, by tetrameric MHC-peptide complex analysis (171; 172) the numbers of virus specific T cells during primary infection were shown to be much greater than previously appreciated. The present data suggest that the numbers of different T-cell clones involved in responses to tumour antigens may be of unexpected high magnitude. Whether the high number of T-cell clonotypes in the melanoma lesions reflects the recognition of multiple different melanoma associated peptide antigens remains elusive. It could be speculated that the list of melanoma antigens recognised by T cells characterised so far is not yet complete. In this respect it is interesting that most antigens characterised to date have been identified using *in vitro* expanded TIL. Recent data suggests that *in vitro* culture may eliminate prominent *in vivo* T-cell populations indicating that *in vitro* TIL inadequately reflects the *in vivo* situation (106; 173). Furthermore, to our knowledge, no compelling data are available with respect to antigens recognised by T cells *in situ*.

The high number of clones present in the lesions prompted us to investigate in detail the location and area of distribution of specific T-cell clones in the lesions. Immunohistology revealed the presence of numerous T cells throughout the tumour (data not shown). However, the use of specific antibodies against TCRBV regions clearly demonstrated that the T-cell clones expressing these regions were located as clusters of cells (Fig. 14) suggesting a local clonal expansion.

To investigate further whether T cells clonally expanded *in situ* were local by nature, we compared the clonotypes detected in the different lesions from each patient, with the aim of resolving whether specific T-cell clones were identical, i.e. originated from the same clonal expansion. We therefore employed TCR clonotype mapping for a comparative analysis of specific T-cell clonotypes in the different lesions. The results demonstrated that the preponderance of the clonotypes were exclusively

located in a single lesion only (Fig 14). The *in vivo* accumulation of the same T-cell clone in different metastases has been reported (169; 174). The present study corroborates these findings, as a minority of the detected T-cell clonotypes was present in two lesions (Fig. 13). Nevertheless, our data indicate that the vast majority of the T-cell clones do not accumulate at several metastatic sites.

These data, together with the fact that clonotypic T cells were not detected in the blood samples, indicate that the T-cell response is initiated and executed by local T-cells that become activated and expand clonally, but do not enter the periphery. Alternatively, it is possible that clonally expanded T cells, if they enter the periphery, have been through multiple cell cycles and therefore reach an anergic or senescent state upon arrival to other metastatic lesions.

The general conclusions from the present study are that the T-cell infiltrate of malignant melanoma is exceedingly heterogeneous and contrived by a much higher number of different clonotypic T cells than previously appreciated. Furthermore, our data indicate that the vast majority of clonotypic T cells in melanoma TIL, at least in the case of subcutaneous metastasis, may be considered as strictly local.

Clonotypic T-cell expansion induced by melanoma: comparison of the *in vivo* and *in vitro* situation

Abstract

Melanoma is generally accepted as being antigenic and capable of eliciting T-cell responses, which however, in most cases are inadequate to control tumour growth. Several studies have focused on the analysis of TIL and demonstrated overexpression of specific TCR variable regions due to clonal expansions of T cells. We recently reported the development of a new technique based on RT-PCR and DGGE, which allows the detection of clonally expanded T cells with high sensitivity. Using this method we were able to demonstrate that the T-cell infiltrate in melanoma is exceedingly heterogeneous and contains a higher number of T-cell clonotypes than previously appreciated.

Here, we report the analysis of a subcutaneous melanoma metastasis and a cytotoxic T-cell line, TIL92, derived from this lesion, for the presence of clonally expanded T cells. A high number of T-cell clonotypes was demonstrated in the tumour, whereas TIL92 consisted of only a limited number of T-cell clones. Furthermore, the majority of the T-cell clonotypes detected in the lesion were different from those expanded in TIL92. Our data indicate that the heterogeneity of the T-cell infiltrate is lost during *in vitro* culture and that standard culture conditions favour the outgrowth of clonotypes, which do not necessarily participate in the *in vivo* response against melanoma.

Introduction

There is a consensus that T cells play a major role in the control of melanoma (175). This consensus is based on the demonstration of tumour-specific T cells and a number of clinical observations, e.g., the spontaneous regression of primary tumours and the induced regression of metastatic lesions subsequent to immune modulatory therapies. Recently, the fine specificity of *in vitro* expanded anti-melanoma T cells has been established and the recognised tumour antigens have been defined (176). The characterisation of these antigens has, however, demonstrated the genuinely low immunogenicity of most of them. Thus, the question of whether *in vitro* expanded T cells reflect the *in vivo* immune response has been raised (106). So far it has not been possible to answer this question due to methodological problems in characterising the *in vivo* T-cell response. Recent technological advances such as the MHC class I tetramer technique (171) or the TCR clonotype mapping (166) have shed unexpected light on the nature of T-cell responses to pathogens and tumours. The latter technique enabled us to demonstrate that the T-cell infiltrate in melanoma is exceedingly heterogeneous and contain a higher number of T-cell clonotypes than previously appreciated (177).

In the present study we have addressed the question of whether clonal T-cell expansions occurring *in situ* are maintained *in vitro*, using standard culture conditions.

Materials and Methods

Cell culture

The FM92 melanoma cell line was established from a subcutaneous metastasis processed immediately after surgery. Serological tissue typing was performed (HLA-A1, A2, B8, B44, Cw3, Cw5, DR3, DR9), and FCM analysis demonstrated that the tumour cells had retained expression of the HLA class I molecules (109). Further analysis demonstrated that MART-1/Melan-A and gp100 were expressed in the tumour lesion as well as in FM92, whereas mRNA for tyrosinase was not detectable.

The cytotoxic T-cell line (CTL), TIL92, was established by placing mechanically dispersed tumour fragments in 24-well plates (Nunc, Denmark) in RPMI-1640 medium containing 10% FCS. Three days later, 20 U/ml Interleukin 2 (IL-2) and 10 U/ml Interleukin 4 (IL-4) were added. The addition of cytokines was repeated every 3-4 days. Cytotoxic activity directed against the autologous melanoma cells was present after 10 days of culture (data not shown). Cultures were expanded when cell densities reached 2 x 10^6 per well. The cells used in the present study had been in culture for 5 weeks, at this timepoint they exhibited strong cytotoxicity against the autologous melanoma cell line FM92 (data not shown).

TCR clonotype mapping

Tumour material and blood samples were processed immediately after surgery. RNA was extracted using the Purescript Isolation Kit (Gentra Systems Inc. NC). cDNA synthesis and quantitation of TCR cDNA in the each sample were carried out as described (136). Using equal amounts of TCR template in all reactions, cDNA was amplified using primers specific for TCRBV regions 1-24 together with the "GC-clamped" constant region primer (166). Amplifications were carried out in a total volume of 25 µl containing 1xPCR buffer (50mM KCl, 20 mM Tris pH 8.4, 2.0 mM $MgCl_2$, 0.2 mM cresol red, 12% sucrose, 0.005% (w/v) BSA (Boehringer-Mannheim, Mannheim, Germany)), 2.5 pmol of each primer, 40 mM dNTPs (Pharmacia LKB, Uppsala, Sweden) and 1.25 units of AmpliTaq polymerase (Perkin Elmer Cetus Corporation, Emeryville, CA.). The parameters used for amplification were 94°C for 30 sec, 60°C for 30 sec. and 72°C for 30 sec. for 40 cycles. Taq polymerase and dNTPs were

added to the reaction tube at a 80°C step between the denaturation and annealing steps of the first cycle. Gels for DGGE analysis contained 6% polyacrylamide and a gradient of urea and formamide from 20% to 80%, and were run at 160 V for 4.5 h in 1x TAE buffer kept at a constant temperature of 58°C. After electrophoresis, the gel was stained with ethidium bromide and photographed under UV transillumination.

Sequencing reaction

Sequence analysis of PCR products was performed using the Thermo Sequenase cycle sequencing kit (Amersham, Life Science, Cleveland, OH) according to the manufacturer's instructions. In brief, bands were excised from the denaturing gradient gel, and DNA was eluted in H_2O and reamplified. An aliquot (0.2 µl) of the PCR product was used as template in a 40-cycle sequencing reaction with ^{33}P labeled constant region sequencing primer. Gels were dried under vacuum and exposed to a Phosphor Screen.

Results

Peripheral blood lymphocytes and a subcutaneous melanoma lesion were subjected to RT-PCR/DGGE clonotype mapping. The analysis of PBL revealed very few, faint bands within a background smear, suggesting only limited clonal expansions of T cells (Fig. 15A). In contrast, the tumour was characterised by the presence of multiple T-cell clonotypes (Fig. 15B). Clonotypic TCR transcripts were demonstrated for the majority of the BV regions. In most cases, several clones was identified for each individual BV family. However, for a few BV regions, e.g. BV10, BV11 and BV24, only a single clonotype was detected (Fig. 15B).

TIL propagated *in vitro* often show restricted TCRBV repertoire. In order to examine whether the dominant clones isolated *in vitro* were representative of the *in vivo* accumulation of T cells within the tumour, we performed TCR clonotype mapping of the CTL line TIL92 which was established from the melanoma lesion. The results from this DGGE analysis (Fig. 15C) differed substantially from the results obtained from the analysis of the tumour lesion. TIL92 showed a low degree of heterogeneity as compared to the tumour lesion, i.e., only a single T-cell clone was present for each BV region in most cases. Furthermore, the clonotypes for BV2 and BV5 present in TIL92 were completely absent in the tumour lesion; *vice versa* the clonotype for BV10 was present *in vivo*, but was lost during *in vitro* propagation.

These observations prompted us to test whether the clonotypes detected in TIL92 were identical to those present in the tumour from which TIL92 was established. For this purpose we repeated the RT-PCR analysis and loaded samples onto a denaturing gradient gel in adjacent lanes. This direct comparison of the TCR clonotypes present in the tumour and TIL92 demonstrated that very few of the clonotypes were identical (Fig. 16). The BV regions BV14, BV16, BV20 and BV24 were subjected to sequence analysis to verify potential sequence identity. In all cases sequence identity was verified (data not shown).

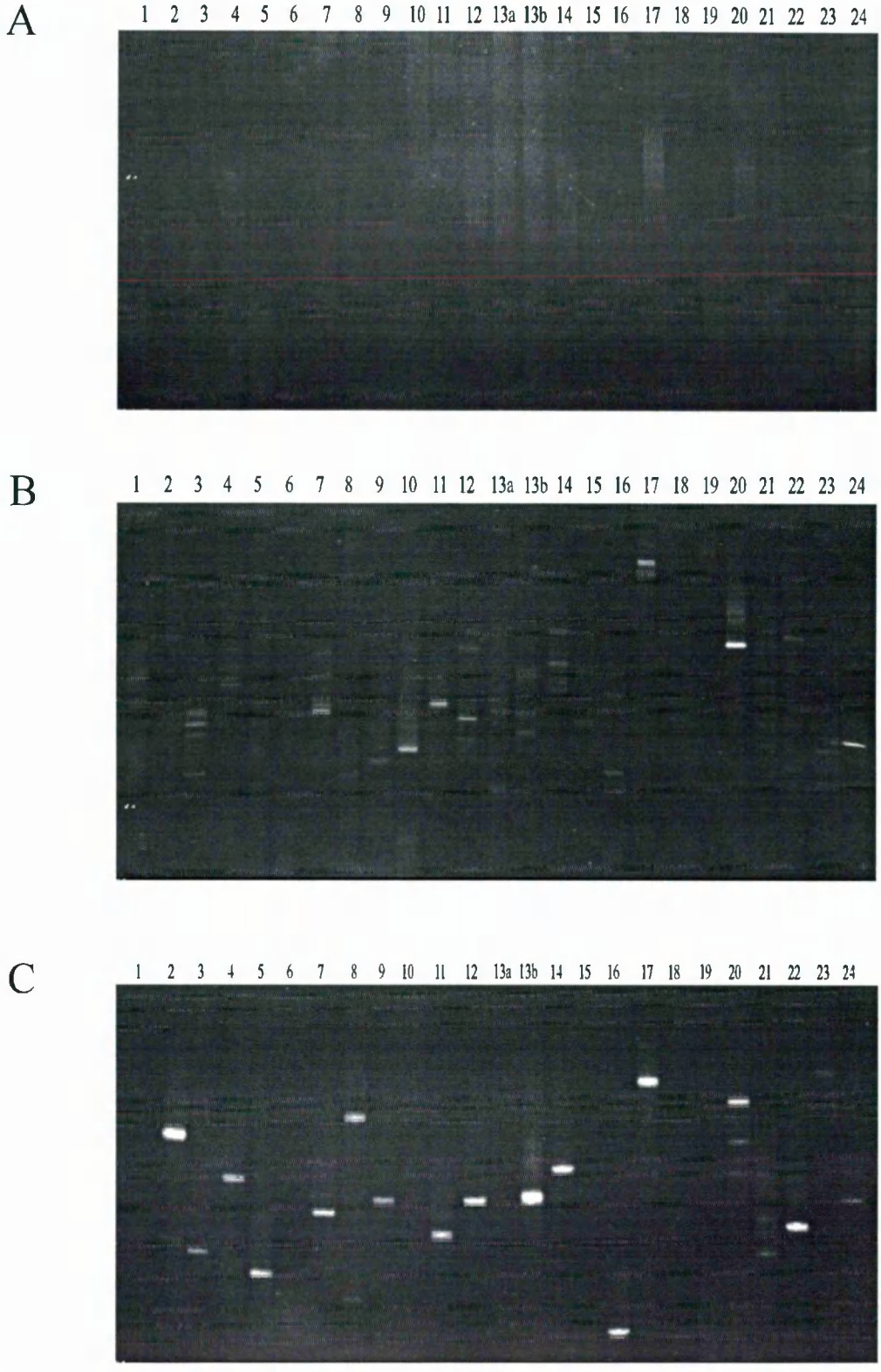

Figure 15. TCR clonotype maps of PBL, tumour biopsy and TIL92. cDNA from tumour tissues and peripheral blood lymphocytes were amplified with primers specific for BV families 1-24 and aliquots loaded onto a 20% - 80% denaturing gradient gel and run for 4.5 hours at 160 Volts at a constant temperature of 58°C. DNA was stained with ethidium bromide and photographed under UV light. A, PBL; B, Biopsy; C, TIL92.

Discussion

Antigens recognised by CD4 and CD8 T cells have been identified on melanoma cells (178). Previous studies have suggested that antigen-selected TCR repertoires may vary in complexity from very limited to extremely diverse. These differences reflect at least in part methodological problems in characterising the *in vivo* T-cell response. Although substantial progress in the characterisation of T-cell responses against viruses was made through the introduction of the MHC class I tetramer technique (171), the characterisation of T-cell responses against melanoma still largely depends on the *in vitro* expansion of T cells. Recent reports demonstrated that tetrameric complexes are suitable to detect and isolate T cells recognising known melanoma associated antigens (72). However, characterisation of the known antigens revealed the genuinely low immunogenicity of most of them (178). The low immunogenicity hampers the design of suitable tetrameric complexes or the application of other highly sensitive detection methods, e.g. the IFN-γ ELIspot assay to determine the complexity of the T- cell response. Still, these new techniques posed the question about the value of *in vitro* expansion techniques such as limiting dilution cloning since the results obtained suggested a more heterogeneous T-cell response than hitherto predicted.

In the present study we probed the question whether this *in vivo* heterogeneity of the T-cell infiltrate is maintained under standard *in vitro* culture conditions. A subcutaneous melanoma metastasis and a cytotoxic T-cell line, TIL92, established from this metastasis were analysed for the presence of clonally expanded T cells. A complex and highly diverse pattern of T-cell clonotypes was demonstrated in the melanoma lesion, in contrast to TIL92 in which only a limited number of clonotypes were detected. Furthermore, the vast majority of the T-cell clonotypes detected in the tumour lesion were shown to be different from those present in TIL92.

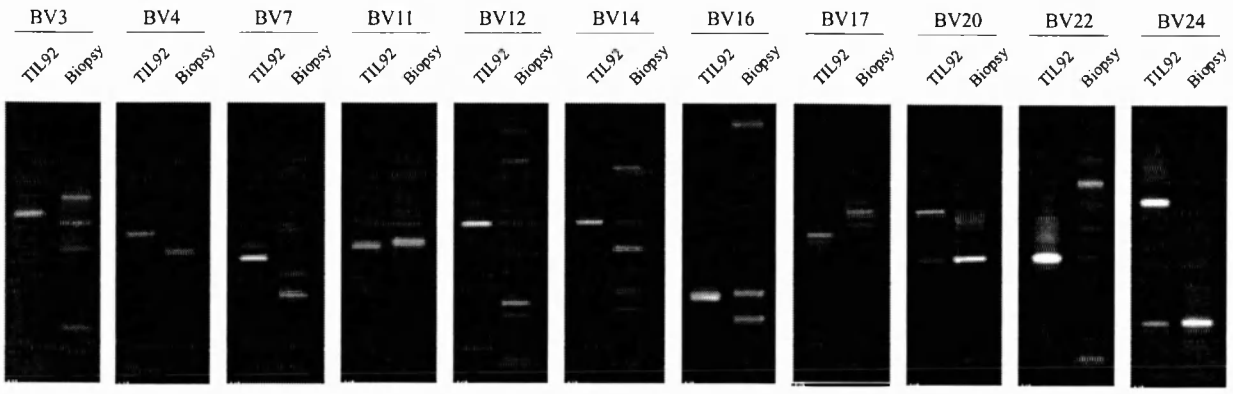

Figure 16. Comparative DGGE analysis of T-cell clonotypes in tumour biopsy and TIL92. To compare the T-cell clonotypes in biopsy and TIL92, amplified cDNA were loaded onto a 20% - 80% denaturing gradient gel in adjacent lanes, and run for 4.5 hours at 160 Volts at a constant temperature of 58°C. DNA was stained with ethidium bromide and photographed under UV light.

In corroboration with the data presented here, several recent studies raised the concern whether the T-cell clones isolated *in vitro* are representative of the population within the tumours, rather than being a reflection of an *in vitro* selection (106; 173). The latter has been indicated from dramatic changes in the BV region repertoire upon *in vitro* culture (144). We observed the appearance of specific BV regions in TIL92, i.e., BV2 and BV5, not present in the tumour lesion (Fig 15, B and C). However, several BV regions detected in TIL92 were also present in the tumour lesion, including BV3, BV4, BV7, BV11, BV14, BV16, BV17, BV20, BV22 and BV24. Dufour *et al.* reported the *in vitro* propagation of a T-cell clone sharing the same BV region as detected *in vivo* by means of the immunoscope approach (179). However, the direct proof of clonotypic identity lacked in this study since no sequence data were provided.

The dramatic clonotypic changes demonstrated here by RT-PCR/DGGE based TCR clonotype mapping indicate that analysis of the BV region usage alone does not reliably reflect the true extent of the changes induced by *in vitro* culture. This observation may reflect that a large proportion of tumour-specific T cells become anergic in the tumour microenvironment (180; 181) and consequently become overgrown by the remaining T cells activated during *in vitro* culture conditions. Alternatively, clonotypes detected in tumour specimens represent T-cell clones, which have undergone multiple cell divisions. These clones are more likely to reach senescence during *in vitro* culture than T cells that have not been clonally expanded *in vivo* (182).

In conclusion, the maze of the *in vivo* T-cell response against melanoma may be lost during *in vitro* culture. This raise the question of whether tumour associated antigens identified by means of *in vitro* expanded T cells represent tumour regression antigens relevant for the *in vivo* situation.

Concluding remarks and perspectives

Since the first reports on clonotypic *in vivo* T cells in melanoma were published some five years ago (102; 183), the concept of the specific recognition of melanoma associated antigens by T cells has become generally approved. The present Thesis adds new insight into the nature of the T-cell response against melanoma.

The data presented in paper 1 demonstrate that clonotypic T cells are present in both regressive and progressive parts of the same primary melanoma lesions. Using the DGGE based methodology, we demonstrate in paper 3 that the T-cell infiltrate in melanoma is exceedingly heterogeneous and includes a much higher number of T-cell clonotypes than previously appreciated. Furthermore, paper 3 indicates that the vast majority of these T-cell clonotypes are strictly local in their distribution. In paper 4 we demonstrate that normal *in vitro* culture conditions do not support the growth of *in vivo* expanded T cells.

The specific aim of paper 1 was to investigate whether the progressive parts of tumours would give rise to clonal T-cell responses. Loss of HLA molecules is generally accepted to be a means of immune escape used by melanoma cells and the lack of clonal T-cells in progressive parts of the tumour would support this assumption. Our data, however, suggest that HLA restricted T-cell responses do take place in both regressive and progressive parts of the lesions indicating that loss of molecules involved in the presentation or processing of antigens is an infrequent mechanism of immune escape in melanoma. Nevertheless, melanomas may in some cases lose the expression of class I molecules. Interestingly, in cell lines established from patients undergoing immunotherapy the losses HLA expression is more frequent (184), supplying indicative evidence to the concept of immune surveillance by T cells.

The study described in paper 1 was performed by analyses of TIL in primary melanoma lesions. Studies investigating the mechanisms of immune escape in melanoma are often conducted using late stage metastatic lesions or melanoma cell lines established from metastatic lesions (185-187). It could be speculated that *in vitro* cell lines are exposed to an immunological selection during establishment, which is quite different from the immunological selection pressure *in vivo*. The

outgrowth of T-cell clonotypes *in vitro,* which were not expanded *in situ* could indicate such a scenario (106; 173; 188). Melanoma cell lines may therefore not be suited for such studies. In some cases the same applies to the use of lesions from late stage melanoma patients. In this respect it is interesting that the expression of Fas ligand by melanoma cells, initially reported to be expressed in 7 out of 7 melanoma lesions (189), has recently been found to be expressed, and only weakly, in only a fraction of primary tumours (10%) (190). Although these data do not imply that FasL expression by melanoma cells is not involved in immune escape, it should be kept in mind that T cells have other means of killing than the Fas/FasL system and that the mechanisms used by TIL *in situ* are not known. Recently, it was shown that *in vitro* CTL clones are resistant to FasL induced apoptosis and that these clones mediate cytotoxicity independent of Fas/FasL ligation (191).

One of the suggested immune escape mechanisms in melanoma is the downregulation of the ζ-chain of the TCR/CD3 complex. Only a single study of the ζ-chain level in TIL and PBL in melanoma patients has been published, and all patients included in this study were suffering from metastatic melanoma (110). It could be speculated that the downregulation of the ζ-chain may not be directly accomplished by the tumour but could be a more general characteristic of long lasting immune responses that are unsuccessful in clearing the antigen from the body, inducing a state of chronic inflammation (192; 193). If this is the case, downregulation of the ζ-chain is secondary to immune escape, merely being the result of having an unsuccessful ongoing immune response.

The model of partially regressing melanoma offers an ideal system to investigate the nature of early events in successful and unsuccessful immune responses against melanoma. Future plans using this model include investigations into the level of Th1/Th2 cytokines (194; 195), the expression of killer inhibitory receptors (KIRs) by TIL of melanoma (81; 196) and the expression of the inhibitor of NK cell lytic activity, HLA-G, by melanoma cells (197).

The high number of T-cell clonotypes in melanoma lesions (as demonstrated in paper 3) by far exceeds the number of clonotypes previously detected in melanoma and other tumours (161; 169). Most analyses have been carried out by using the "Immunoscope" technique. This method reveals T-cell clones by the detection of TCR transcripts which have the same length. As with DGGE/TCR

clonotype mapping, the "Immunoscope" technique is initiated with a specific amplification of each BV family. The size distribution of the DNA molecules withheld in each PCR product is at this point evaluated, and only PCR products having a restricted length pattern are further analysed. Consequently, this approach may not be suitable for the analysis of highly heterogeneous infiltrates. As it appears from the data obtained with the DGGE/TCR clonotype mapping (Fig. 12, page 57 and Fig. 15C, page 68), each BV family may repudiate a number of clonotypic TCR transcripts of unknown length. It could be speculated that the size profile of these PCR products does not differ much from the profile obtained from the analysis of PBL, thereby leaving clonotypic transcripts undetected. Both methods do, however, have their weak points. Using TCR clonotype mapping, different clonotypic transcripts with identical melting properties resolve at the same position in the gel, thereby underestimating the number of clonotypes. By using the "Immunoscope" method, TCR transcripts of identical length will be revealed as a single transcript. However, in contrast to the "Immunoscope" method, the TCR clonotype mapping will not leave clonotypic transcripts undetected.

Our data do not discriminate between CD4 and CD8 cells, but it is well known that both cell types infiltrate melanoma lesions (198). Most likely, the T-cell clonotypes of the infiltrate therefore consists of both CD4 and CD8 T cells. The high degree of heterogeneity and the local nature of the T cells indicates that each T-cell clone does not expand to as high cell numbers as seen in T-cell responses against non-self antigens, i.e. virus infections (172; 199). Whether this reflects the site of infection/metastasis or the nature of the antigens (non-self/self) is not known, but the frequent development of lymph node metastases without the presence of high numbers of MAA specific T cells in the blood (200) indicates that the nature of the antigen is important; non-self antigens being capable of eliciting a much stronger T-cell response than self antigens. Although the number of viral peptide antigens presented on an infected cell during infection probably exceeds the number of melanoma peptides on a melanoma cells, it is conceivable that TCRs with high affinity against dominant self-determinants, binding with high affinity to the HLA molecule, have been negatively selected against in the thymus. It can therefore be speculated that the T-cell response against melanoma is directed against determinants that are only present at rather low levels on the cell surface of the melanoma cell. Although highly efficient cytotoxic T cells may be established *in vitro* against such epitopes, it should

be kept in mind that peptide recognition is much more efficient in the effector phase than in the phase of induction. Furthermore, it is possible that the *in situ* expression of some antigens is lower than their expression *in vitro*. It is therefore conceivable that some *in vivo* TIL are only partially activated due to a low amount of (sub-dominant) antigen on the cell surface.

As mentioned in the Introduction of this Thesis, a high number of MAA have been characterised that theoretically would give rise to an efficient T-cell response. The reason for the lack of such a response could very well differ from antigen to antigen and therefore involve a number of different mechanisms. Regarding the apparently low immunogenecity of proteins of the MAGE family, this was recently suggested to be caused by structures in these proteins that are resistant to proteolytic degradation, thereby hindering formation of the antigenic peptide in sufficient amounts (178).

For the MART-1_{27-35} peptide it has been indicated that molecular mimicry from other self-proteins gives rise to peptide antagonists and partial agonists and that these epitopes are involved in the maintenance of the CTL but are impairing the anti-tumour response (201).

In conclusion, data are lacking that prove the prevalent use of immune escape mechanisms at an early stage in melanoma progression, and most antigens characterised are of low immunogenecity. Possibly the reason for the rarity of HLA losses, antigen losses and other suggested mechanisms of immune escape is that the immune response for a number of reasons is too weak and fails to promote the development of these variants.

The high degree of heterogeneity of the T-cell infiltrate as demonstrated in paper 3 indicate that a high number of different epitopes are being recognised. It is now generally accepted that several different TCRs may be utilised that recognise the same peptide epitope, but the presence of more than 30 different clonal T-cell expansions in each lesion nevertheless indicates a broad spectrum of antigens. Equally striking is the local nature of the vast majority of these clones. The recent development of the MHC class I tetramer technique (171) has been used for the detection of MAA specific T cells in the blood (72). However, the local nature of the T-cell clones as demonstrated in the paper 3 raises the question of whether clonotypes detected in the blood are in all cases relevant to the *in situ* recognition of tumour cells.

An important issue that needs further study is to resolve the question of which antigens are recognised by TIL *in situ*. *In vitro* studies using CTL lines established from TIL or PBL seem to indicate that lines specific for melanocyte differentiation antigens are much easier to establish than lines specific for cancer/testis specific antigens i.e. the MAGE family of proteins. The involvement of melanocyte differentiation antigens in the *in situ* recognition is also indicated by the depigmentation observed in some patients receiving immunotherapy (202). Recently we have used the DGGE methodology to demonstrate the *in vivo* accumulation of the same T-cell clone in a primary melanoma and a vitiligo-like halo about the tumour likewise indicating the involvement of T cells recognising normal melanocytes (203). However, apart from these indications as to the group of antigens involved, the specific targets of *in vivo* recognition have not been determined.

The data presented in paper 4 demonstrate that standard *in vitro* culture conditions of TIL do not support the growth of *in situ* expanded T cells. The complete loss of the *in vivo* heterogeneity of the T-cell infiltrate during *in vitro* culture clearly illustrates the differences between *in vitro* and *in vivo* conditions, and raises the question of whether antigens characterised using *in vitro* CTL are relevant for the *in vivo* situation. Future studies will therefore focus on the establishment of *in vitro* culture conditions that allow the growth of *in situ* expanded T cells and the use of these T cells for subsequent determination of the antigens recognised by T cells *in situ*.

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